

1 **Main Manuscript for**

2 ***CircSry* regulates spermatogenesis by enhancing γ H2AX expression**
3 **via sponging miR-138-5p**

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22 **Keywords:** *circSry* spermatogenesis meiosis miR-138-5p γ H2AX

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25

26 **Abstract**

27 *Sry* on the Y chromosome is the master switch in sex determination in mammals. It has been well
28 established that *Sry* encodes a transcription factor that is transiently expressed in somatic cells of
29 male gonad, inducing a series of events that lead to the formation of testes. In the testis of adult
30 mice, *Sry* is expressed as a circular RNA (circRNA) transcript, a type of noncoding RNA that
31 forms a covalently linked continuous loop. However, the physiological function of this *Sry* circRNA
32 (*circSry*) remains unknown since its discovery in 1993. Here we show that *circSry* is mainly
33 expressed in the spermatocytes, but not in mature sperms and Sertoli cells. Loss of *circSry* led to
34 the reduction of sperm number and the defect of germ cell development. The expression of
35 γH2AX was decreased and failure of XY body formation was noted in *circSry* KO germ cells.
36 Further study demonstrates that *circSry* regulates H2AX mRNA indirectly in pachytene
37 spermatocytes through sponging miR-138-5p. Our study demonstrates that, in addition to its well-
38 known sex-determination function, *Sry* also plays important role in spermatogenesis as a circRNA.

39 **Introduction**

40 Long non-coding RNAs are relatively abundant in the mammalian transcriptome (1), and play
41 important roles in gene regulation in development and reproduction (2). Circular RNA (circRNA) is
42 a unique type of non-coding RNAs generated through back-splicing to form a covalently linked
43 loop (3, 4). Since the first circular RNA was discovered in 1970s (5), very few circRNAs had been
44 identified in the following years. In the last decade, however, the development of RNA
45 sequencing technologies and bioinformatics has greatly facilitated the discovery of circular RNAs
46 (6). Many circular RNAs were found stably expressed in various cell types, and engaged in
47 regulating various biological processes, such as transcription, alternative splicing, chromatin
48 looping, and post-transcriptional regulation (7-12). One of the functional mechanisms of circular
49 RNA is that they act as competing endogenous RNAs (ceRNAs) to sponge miRNAs, therefore
50 regulating gene expression (4, 13-15).

51 *Sry* is best known as the sex-determination gene on Y chromosome. In mouse, *Sry* is
52 expressed as a transcription factor from 10.5 to 12.5 post-coitum (dpc) in the genital ridge
53 somatic cells, initiating testis development (16). Introduction of *Sry* locus into the female mouse
54 embryo switches the sex to male, while the targeted mutation in male embryos leads to complete
55 male-to-female sex reversal (17-19). Moreover, mutation of *SRY* causes a range of sex-disorder
56 development with profound effects in human (20). Recently, a cryptic second exon of mouse *Sry*
57 hidden in the palindromic sequence was identified and this two-exon *Sry* transcript plays primary
58 role in sex determination (21). *Sry* is also expressed in adult mouse testis as a circular RNA
59 (*circSry*) (22-24). A linear transcript containing long inverted repeats is transcribed from a distal
60 promoter, followed by a back splicing event that covalently links an acceptor splice site at the 5'
61 end to a donor site at a downstream 3' end (Figure 1A) (4). Although the presence of *circSry* in
62 the testis has long been discovered, the significance of *circSry* remains elusive.

63 In this study, we generated mouse models that did not express *circSry*, without interfering with
64 male sex determination. We found that *circSry* played an important role in spermatogenesis, and
65 further dissected the underlying mechanism. Our findings highlight a unique synergy between
66 *Sry*'s male sex-determination role as a protein and its regulatory role as a circular RNA in male
67 germ cells.

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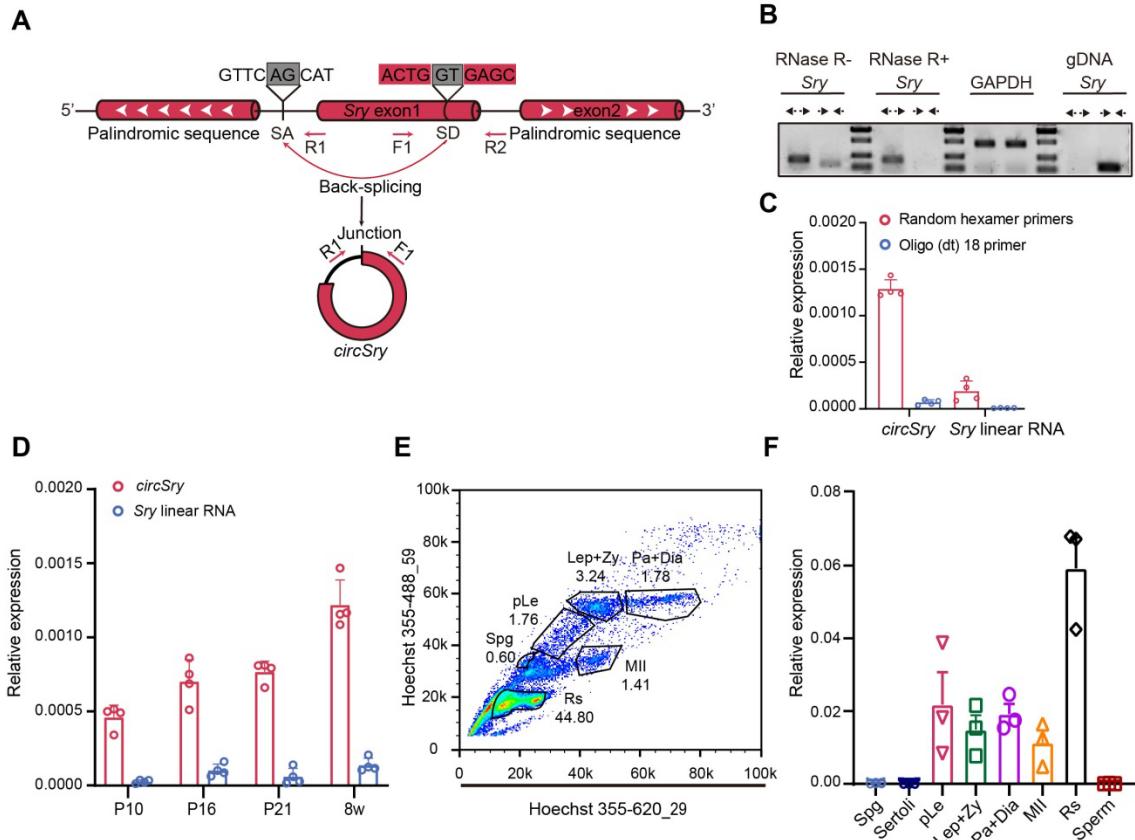
69 **Results**

70

71 **Characterization of *circSry* in mouse testis**

72 To identify *Sry* transcripts in adult mouse testis, divergent primers and convergent primers were
73 designed to amplify *circSry* or *Sry* linear RNA respectively (Figure 1A). Upon RNase R treatment,
74 *circSry* was still detectable by RT-PCR, while linear RNA was not (Figure 1B). The expression of
75 *circSry* was abundant when random hexamer primers were used for reverse transcription, while
76 only weak signal was detected using oligo (dt)₁₈ primers. In comparison, the expression of *Sry*
77 linear RNA was barely detectable using either random hexamer primers or oligo (dt)₁₈ primers

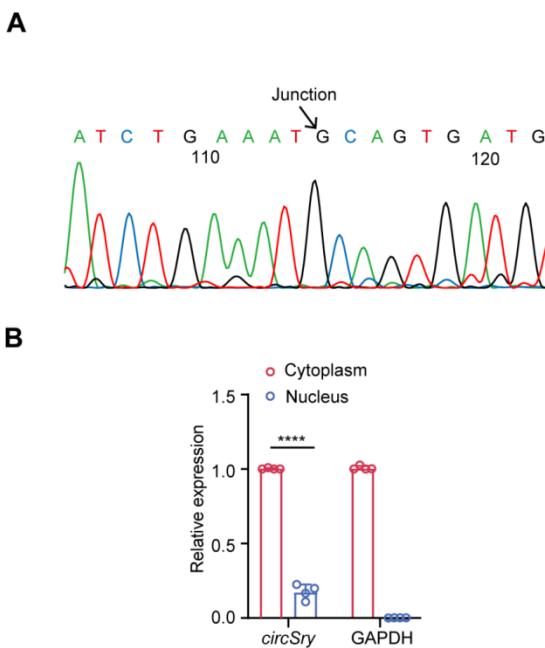
78 (Figure 1C). The presence of head-to-tail splicing site of *circSry* was verified by Sanger
79 sequencing (Figure 1 figure supplement 1A). Furthermore, by separating the cytoplasm and
80 nucleus fractions, we found that *circSry* was mainly localized in the cytoplasm (Figure 1 figure
81 supplement 1B). All these results confirmed previous reports that *Sry* transcripts in adult mouse
82 testis are non-polyadenylated circular RNAs mainly localized in cytoplasm (24).



83 **Figure 1. Identification of *circSry*.** (A) Scheme illustrating of the generation of *circSry*. *CircSry* is formed by an
84 incomplete single exon *Sry* gene through back splicing mechanism. Convergent or divergent primers detect circRNA (F1
85 and R1) or linear RNA (F1 and R2) of *Sry*. Gray box indicates the head-to-tail splicing sequence. (B) Production of
86 divergent primers was resistant to RNase R treatment. *CircSry* was not amplified using genomic DNA as template (n=3);
87 GAPDH was used as control. (C) Random hexamer primer or Oligo (dt)₁₈ primers were used to analyze the expression
88 levels of *circSry* in 8 week-old control mice (n=4). (D) Relative expression of *circSry* or linear RNA in testis from 10 days
89 postnatal to adulthood (n=4). (E) Fluorescence cytometry separated different subtypes of germ cells in adult testis and (F)
90 relative expression of *circSry* in 8 types of cells. (Spg: spermatogonium; pLe: pre-leptotene stage; Lep+Zy: leptotene
91 stage and zygotene stage; Pa+Di: pachytene stage and diplotene stage; MII: meiosis II stage; Rs: round spermatids;
92 Sperm: mature sperm; Sertoli: Sertoli cells; n=3). The data are presented as the mean \pm s.e.m. Source data is available
93 as a Source Data file.
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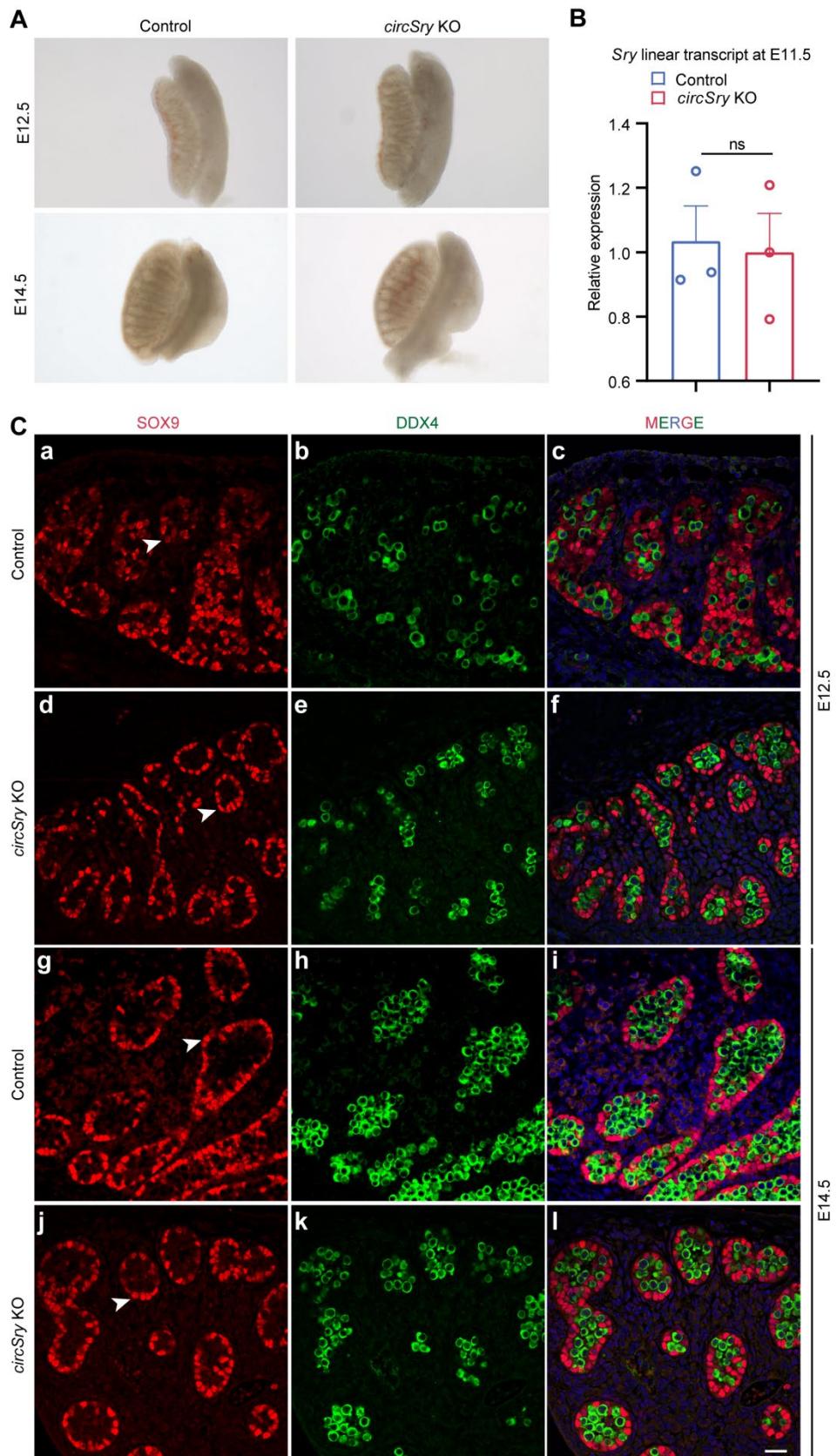
96 Next, we measured the level of *Sry* transcription from day P8 to adulthood. The amount of
97 *circSry* increased over time while linear RNA was barely detectable (Figure 1D). To examine the
98 expression of *circSry* in different cell types of adult testes, different types of germ cells were
99 isolated by flow cytometry (39): spermatogonia (SpG), Pre-leptotene spermatocytes (pLE),
100 leptotene and zygotene spermatocytes (Lep+Zy), pachytene and diplotene (Pa+Di)
101 spermatocytes and round spermatids (Figure 1E). Mature sperms were obtained from adult
102 mouse epididymis and Sertoli cells were obtained from P20 testis. *CircSry* was detected in
103 meiotic and post-meiotic germ cells, expressed at the highest level in round spermatids. There
104 was no *circSry* detected in spermatogonia, mature sperms or Sertoli cells (Figure 1F).



105
106 **Figure 1 figure supplement 1. Characterization of *circSry* in mouse testis (related to Figure 1).** (A) Splicing junction
107 was confirmed by Sanger sequencing. The arrow indicated the head-to-tail splicing site of *circSry*. (B) qRT-PCR results of
108 cytoplasm-nucleus distribution of *circSry* (****P<0.0001; unpaired two-tailed t test, n=4). The data are presented as the
109 mean \pm s.e.m.

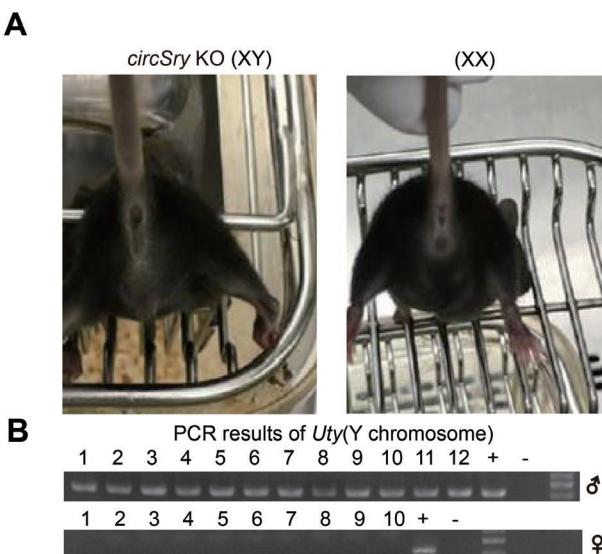
110 **Generation of *circSry* KO mouse**

111 To determine whether *Sry* is involved in spermatogenesis, we generated *circSry* KO mouse via
112 CRISPR/Cas9 (Figure 2A). We designed a sgRNA to specifically target splice acceptor site
113 upstream of *Sry* coding region. Deletion of the 11 bp region harboring splice acceptor site led to



115 **Figure 2 figure supplement 1. No significant difference was detected in *circSry* KO embryos gonads (related to**
116 **Figure 2).**(A) No significant difference was detected in control and *circSry* KO gonads morphology at E12.5 and E14.5 (B)
117 Quantitative analyses of *Sry* gene in control and *circSry* KO gonads at E11.5. The mRNA expression level of *Sry* in in
118 control and *circSry* KO gonads was not significantly changed at E11.5. The gender of the embryos was confirmed with
119 PCR using *Sry* primers. Data are presented as the mean \pm s.e.m; unpaired, two tailed t test, ns, not significant, $p > 0.05$.
120 (C)SOX9 was expressed in Sertoli cells of both control and *circSry* KO gonads at E12.5 and E14.5. SOX9/DDX4 double-
121 staining experiment was performed with control and *circSry* KO embryos at E12.5 (a-f) and E14.5 (g-l). Germ cells were
122 labeled with DDX4 (green). DAPI (blue) was used to stain the nuclei. The arrowheads point to SOX9-positive Sertoli cells
123 (red). The gender of the embryos was confirmed with PCR using *Sry* primers. Scale bars indicate 50 μ m.

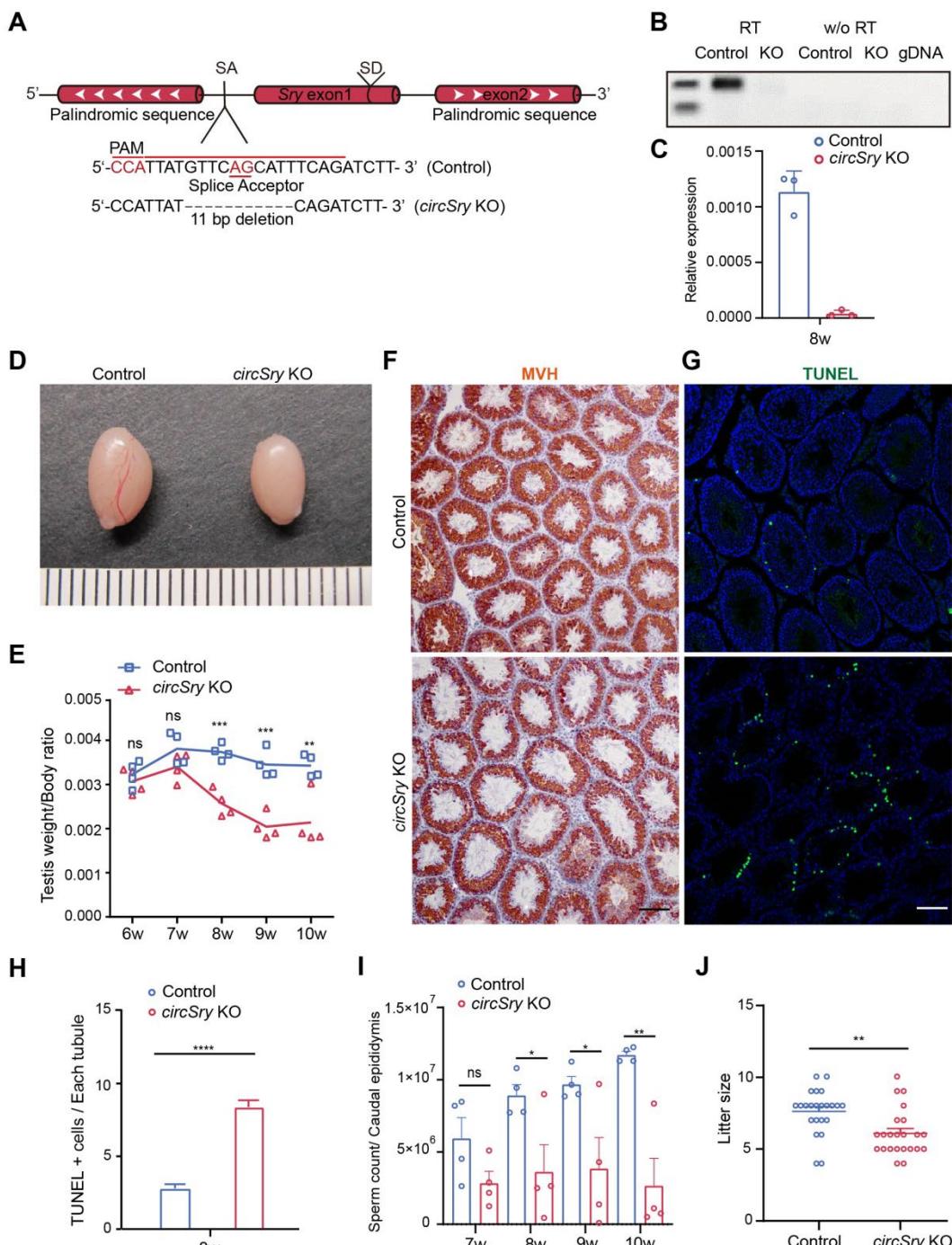
124 complete loss of *circSry* (Figure 2, B and C). No significant difference was detected in control and
125 *circSry* KO embryo gonads (Figure 2 figure supplement 1A-C). No gross abnormalities of external
126 genitalia were observed in 8-week-old *circSry* KO (XY) male founder mice (Figure 2 figure
127 supplement 2A), and they were fertile. In F0 and F1 generation, all the male mice carried Y
128 chromosome and all the females did not, these results demonstrated that this deletion of 11bp
129 upstream region of *Sry* did not interfere with sex determination (Figure 2 figure supplement 2B).
130 Female offsprings were normal, and no developmental defects were observed (Figure 2 figure
131 supplement 2A).



132
133 **Figure 2 figure supplement 2. *CircSry* KO mouse develop as male (related to Figure 2).**(A) F1 offsprings of *circSry*
134 KO developed as male, and offspring XX mice exhibit normal female external genitalia and mammary glands. (B) PCR
135 results of gender identification of F1 off springs. Y chromosomal gene *Uty* was detected by PCR.
136

137 **The fertility of *circSry* KO male mice was affected**

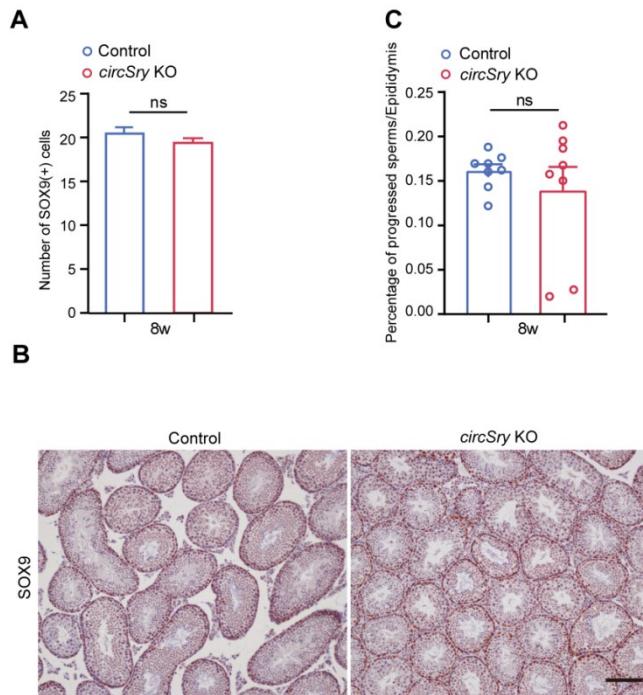
138 To determine the function of *circSry*, we assessed the testes of 6 to 10 week-old males and
139 examined the germ cell development in *circSry* KO mice. The size of testes from *circSry* KO mice
140 was smaller than that of control (wild-type C57BL/6) mice at 8 weeks (Figure 2D). The testis to
141 body weight ratio of *circSry* KO mice was comparable to that of control mice at 7 weeks, whereas
142 it became significantly lower from 8 to 10 weeks (Figure 2E). The development of germ cells was
143 examined by MVH staining. As shown in Figure 2F, the histology of the seminiferous tubules was
144 grossly normal. MVH-positive germ cells were detected in the testes of both control and *circSry*
145 KO mice at 8 weeks (Figure 2F). However, the lumen size of seminiferous tubules in *circSry* KO
146 mice was larger compared with that of control mice (Figure 2F). There were notably more
147 TUNEL-positive apoptotic cells in the seminiferous tubules of *circSry* KO mice than that of control
148 mice (Figure 2, G and H). Furthermore, we found that the total number of sperms in the caudal
149 epididymis of *circSry* KO mice was lower than that in control mice (Figure 2I). To test the fertility
150 of *circSry* KO mice, we crossed 2-3 month-old *circSry* KO male with wild-type female mice and
151 counted the litter size born within 4 months. *CircSry* KO mice produced smaller litter size than
152 age-matched control male mice (Figure 2J). Notably, no difference of SOX9 positive Sertoli cells
153 was detected between control and *circSry* KO seminiferous tubules (Figure 2 figure supplement 3,
154 A and B), suggesting that Sertoli cells were not affected. In addition, the percentage of mobile
155 sperm remained no difference between the control and the *circSry* KO mice (Figure 2 figure
156 supplement 3C), indicating that the loss of *circSry* did not affect sperm mobility. Taken together,
157 these results suggested that the germ cell development was abnormal in *circSry* KO mice.



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Figure 2 Loss of circSry impairs male mice fertility. (A) Design of deleting circSry using CRISPR/Cas9; a specific sgRNA was designed to target splicing acceptor site of circSry. (B) RT-PCR results of circSry. (C) Quantification of circSry within control or circSry KO mice testes. (D) Representative image of circSry KO and control (wild-type) testis of 8-week-old mice. (E) Testis/body ratio of circSry KO and control mice from 6 to 10 weeks of age. P values are presented above the relevant bars (*p<0.05, **p<0.01, ***p<0.001, ns, not significant; unpaired, two tailed t test, n=4). (F) Germ cells were labeled with antibody against MVH (brown). Loss of epithelium within the seminiferous tubules in circSry KO compared with control mice. Scale bars indicate 100 μ m. (G) TUNEL signal in circSry KO and control mouse testis. The total

166 tubule number reached 200 (****p<0.0001; unpaired, two tailed t test). (I) Sperm count of *circSry* KO mice and control
167 mice from 7 to 10 weeks of age (*p<0.05, **p<0.01, ns, not significant; unpaired, two tailed t test n=5). (J) Litter size of
168 *circSry* KO compared with control mice. Random 8-week-old males of control and *circSry* KO were chosen to breed with
169 6-week-old female of C57BL/6 for 4 months (**p<0.01, unpaired, two tailed t test; n=4). Scale bars indicate 100 μ m. The
170 data are presented as the mean \pm s.e.m.



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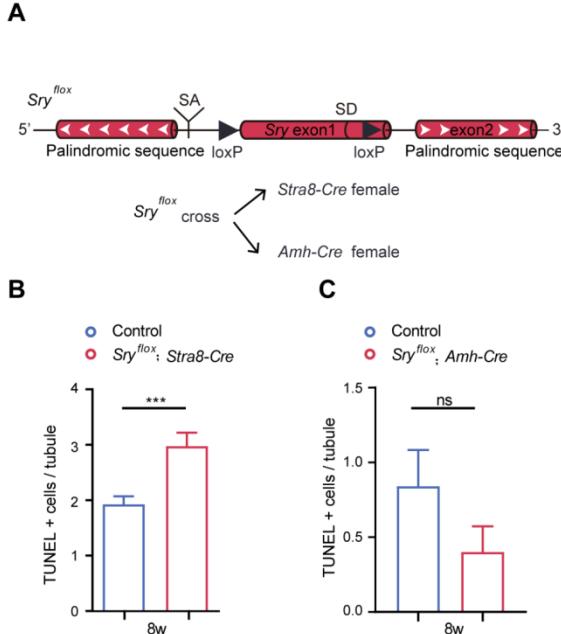
173 **Figure 2 figure supplement 3. Immunohistochemical staining of SOX9 and sperm motility analysis in control and**
174 ***circSry* KO mice (related to Figure 2).** (A) Quantification of progressed sperm obtained from epididymis between *circSry*
175 KO and control mice. (ns, not significant; unpaired, two tailed t test, n=8). Sperms were collected from 8-week old *circSry*
176 KO or control mice epididymis. (B) Representative image of Immunofluorescence staining of SOX9 in 8-week old control
177 and *circSry* KO mice testes. Scale bars indicate 50 μ m. (C) Quantification of SOX9 positive cells within seminiferous
178 tubules from 8-week old control and *circSry* KO mice (ns, not significant; unpaired, two tailed t test, control, n=30; *circSry*
179 KO, n=30). The data are presented as the mean \pm s.e.m.

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181 Germ cell specific knockout of *circSry* led to the defects of spermatogenesis

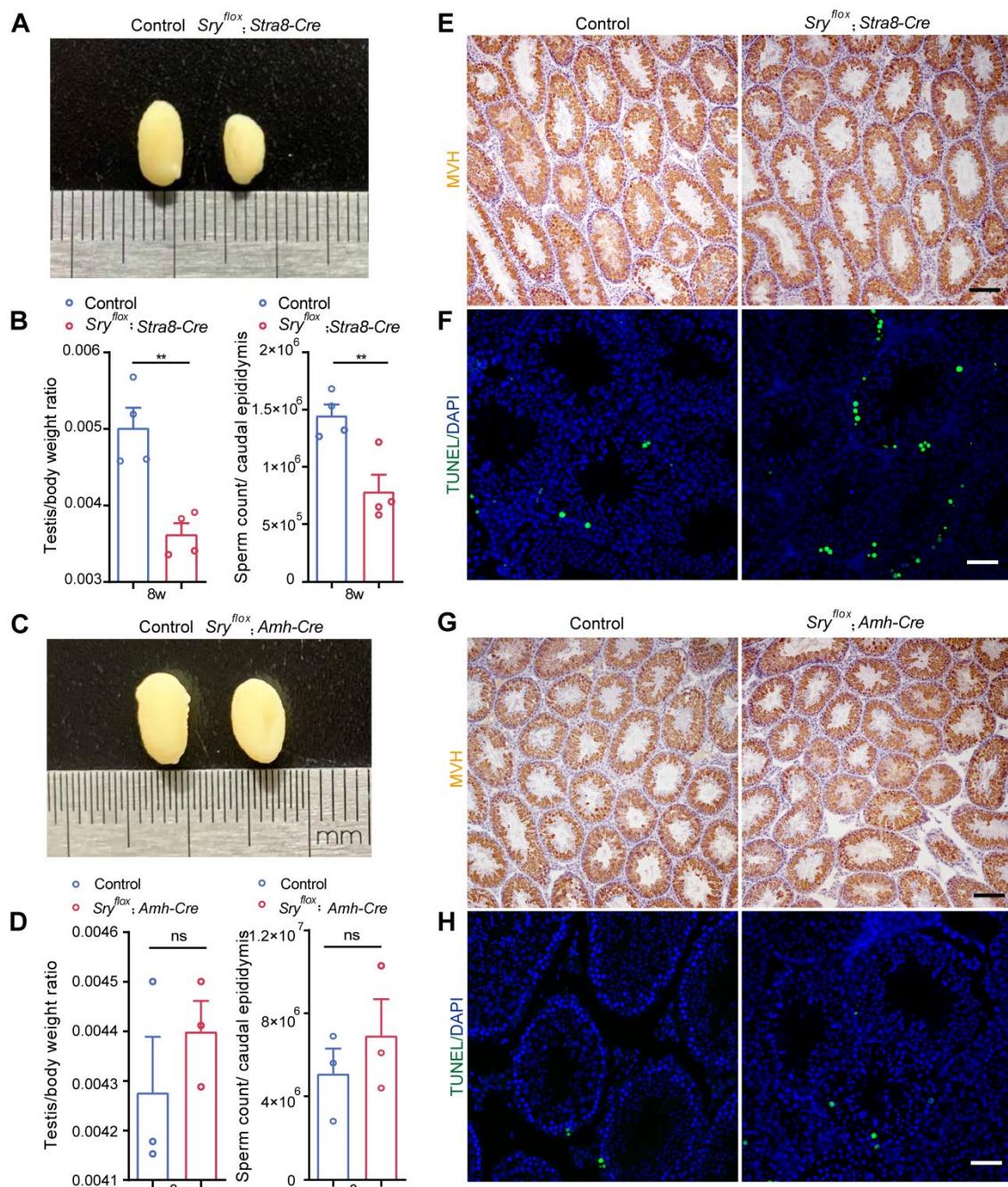
182 To characterize the cell type specific function of *circSry* in spermatogenesis more rigorously, we
183 generated a *Sry* conditional KO mouse model *Sry*^{fl/fl} by inserting two loxP sites flanking *Sry*
184 (Figure 3 figure supplement 1A). *CircSry* was specifically knocked out in germ cells or Sertoli cells

185
186



187 **Figure 3 figure supplement 1. Conditional knockout of *Sry* (related to Figure 3).** (A) Schema of inserting loxP
188 sequences into *Sry* locus. *Sry*^{flox} was mated with *Stra8-Cre* female or *Amh-Cre* female to generate conditional knockout
189 mice in germ cells or Sertoli cells, respectively. (B and C) Quantification of apoptotic cells in *Sry*^{flox}; *Stra8-Cre* or *Sry*^{flox};
190 *Amh-Cre* mice testis seminiferous tubules. The total number of calculated tubules reached 150. (***p<0.001; unpaired,
191 two tailed t test; ns, not significant). The data are presented as the mean \pm s.e.m.

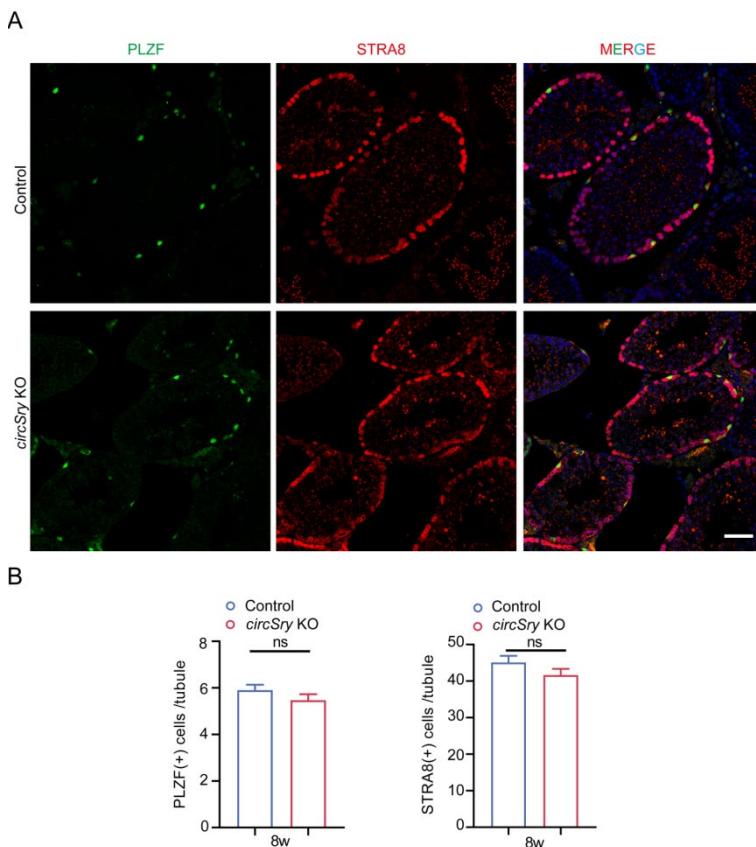
192 by crossing *Sry*^{flox} male mice with *Stra8-Cre* or *Amh-Cre* transgenic mice respectively (Figure 3
193 figure supplement 1A). Compared with control mice, the testes size and the testis to body weight
194 ratio of *Sry*^{flox}; *Stra8-Cre* male was smaller at 2 months of age, and the number of sperms in the
195 caudal epididymis was reduced (Figure 3, A and B). Accordingly, decreased MVH-positive germ
196 cells and increased number of apoptotic germ cells were observed in *Sry*^{flox}; *Stra8-Cre* mice
197 (Figure 3, E and F, Figure 3 figure supplement 1B). These defects were similar to that observed
198 in the *circSry* KO mice. By contrast, no defect of germ cell development was observed in *Sry*^{flox};
199 *Amh-Cre* male mice (Figure 3, C and D, G and H, Figure 3 figure supplement 1C), indicating that
200 *Sry* is not required for Sertoli cells function in adult male testis. Taken together, we conclude that
201 *circSry* plays an important role in germ cell development during spermatogenesis.



202
203 **Figure 3. Conditional knockout of *Sry*.** (A and C) Representative image of 8 weeks testis from Sry^{flox} ; *Stra8-Cre* or
204 Sry^{flox} ; *Amh-Cre* (right) compared with control mice (left) from the same litter. (B and D) Testis/body ratio and sperm count
205 of Sry^{flox} ; *Stra8-Cre* or Sry^{flox} ; *Amh-Cre* compared with control mice from same litter. (**p<0.01; unpaired, two tailed t test,
206 n=4). (E and G) Germ cells were labeled with antibody against MVH. Loss of epithelium within the seminiferous tubules in
207 Sry^{flox} ; *Stra8-Cre* or Sry^{flox} ; *Amh-Cre* (left) compared with control mice (right). Scale bars indicate 100 μ m. (F and H) Tunel
208 assay in Sry^{flox} ; *Stra8-Cre* or Sry^{flox} ; *Amh-Cre* (left) and control mice (right) testis seminiferous tubules. Scale bars indicate
209 50 μ m. The data are presented as the mean \pm s.e.m. Source data is available as a Source Data file.

210 **Loss of *circSry* caused reduction of primary spermatocytes during spermatogenesis**

211 To further characterize the defects of spermatogenesis in *circSry* KO mice, we examined the
212 expression of meiosis-associated genes by immunofluorescence (IF). PLZF positive and STRA8
213 positive germ cells were localized at the periphery of seminiferous tubules, and no difference was
214 detected in the number of either PLZF positive or STRA8 positive germ cells between control and
215 *circSry* KO testes (Figure 4 figure supplement 1, A and B). Notably, the number of SYCP3-



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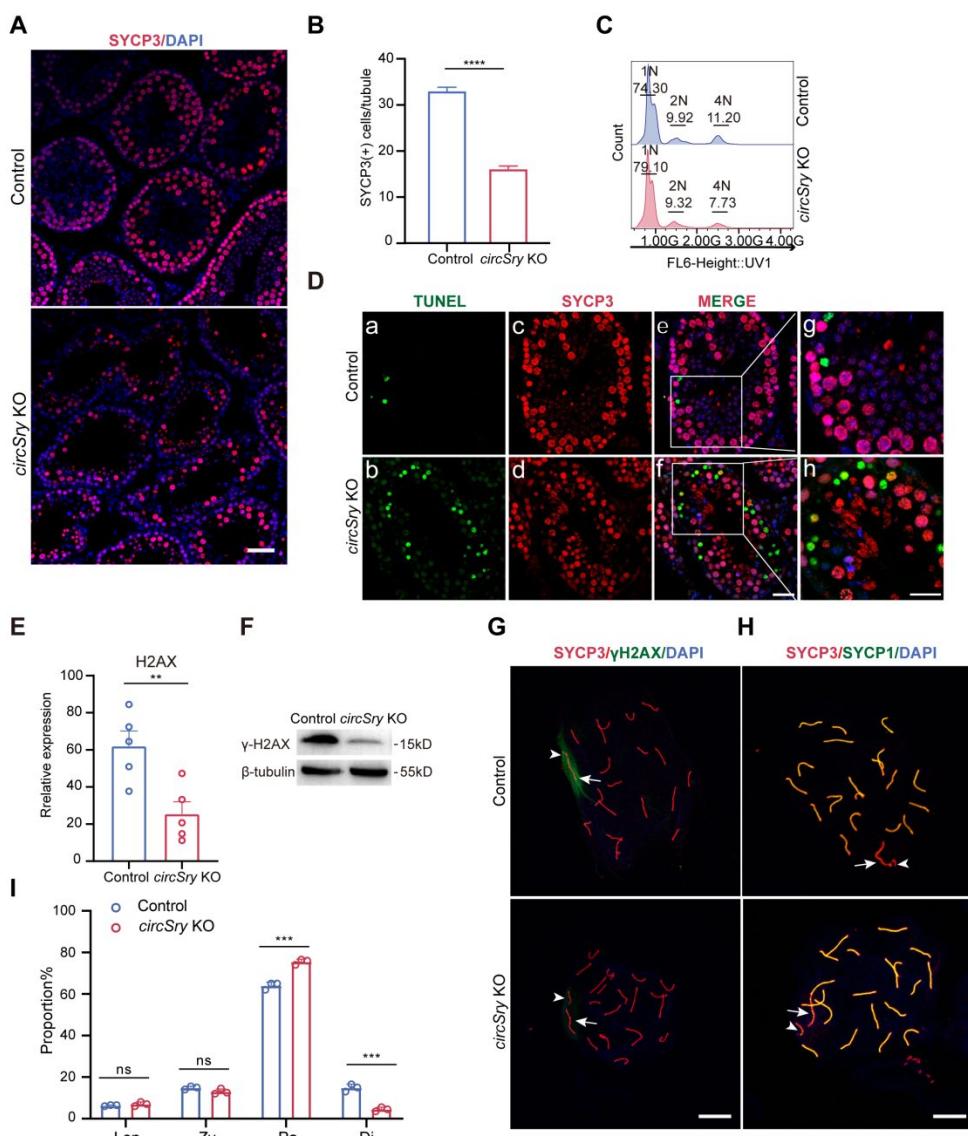
218 **Figure 4 figure supplement 1. Immunofluorescence co-staining PLZF and STRA8 of control and *circSry* KO testes**
219 (related to Figure 4). (A) Representative image of Immunofluorescence staining of PLZF (green) and STRA8 (red) in 8-
220 week old control and *circSry* KO mice testes. Scale bars indicate 50 μ m. (B) Quantification of the number of PLZF positive
221 cells (left) and STRA8 positive cells (right) within seminiferous tubules from three independent mice of 8-week old control
222 and *circSry* KO mice (ns, not significant; unpaired, two tailed t test, control, n=30; *circSry* KO ,n=30). The data are
223 presented as the mean \pm s.e.m.
224 positive germ cells in the seminiferous tubules of *circSry* KO mice was reduced compared with
225 that in control mice (Figure 4, A and B). To assess the progression of spermatogenesis, flow

226 cytometry was used to analyze the proportion of cells with different ploidy levels in 2 month-old
227 testes. The proportion of 4n cells was reduced in *circSry* KO mice, while no difference was noted
228 in the proportion of diploid cells between control and the *circSry* KO mice (Figure 4C). Moreover,
229 TUNEL and SYCP3 double positive germ cells were observed in the *circSry* KO seminiferous
230 tubules, but not in control mice (Figure 4D, a to h). These results suggest that lack of *circSry*
231 leads to defects in meiosis.

232 Since it has been reported that loss of histone γH2AX resulted in aberrant synapsis of sex
233 chromosomes during pachynema, which led to meiotic arrest and apoptosis (25, 26), we
234 conducted chromosome spread experiment of spermatocytes with immunostaining of SYCP3
235 and γH2AX. As shown in Figure 4G, γH2AX expression were decreased in 90% of pachytene
236 stage of *circSry* KO nucleus (n=20) (Figure 4G). To further assess the X-Y synapsis during early
237 pachynema, immunofluorescence staining of SYCP1 and SYCP3 was performed with
238 chromosome spread. In 15.3% of *circSry* KO nucleus (n=300), we observed that X and Y
239 chromosomes paired but not synapsed (Figure 4H). These abnormalities observed in *circSry* KO
240 germ cells were similar with that in *H2ax*^{-/-} germ cells, which led to genomic instability and cell
241 apoptosis (26). Indeed, we observed a significant increase of number of germ cells in pachytene
242 stage and decrease in number of diplotene stage cells (Figure 4I). In contrast, the number of
243 germ cells at leptotene and zygotene stages appeared normal (Figure 4I and Figure 4 figure
244 supplement 3), suggesting that *circSry* functions specifically in pachytene stage.

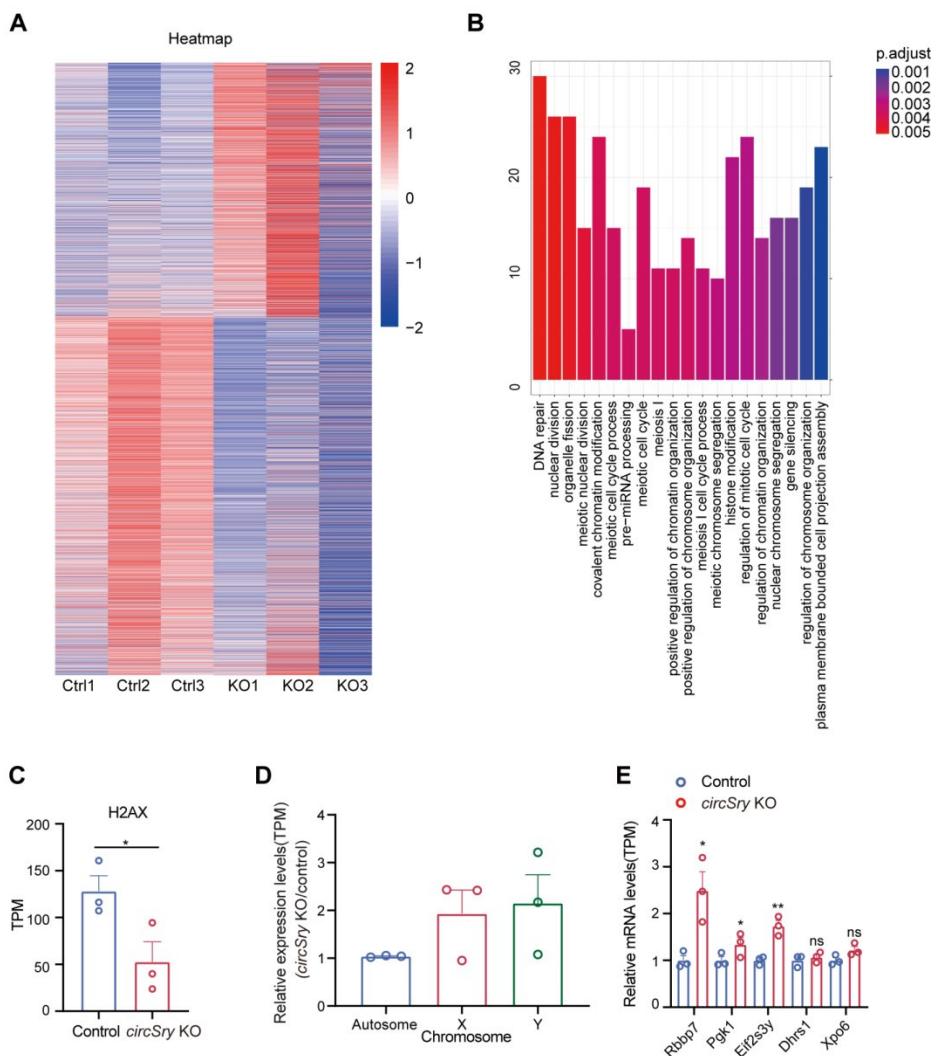
245 Failure of XY body formation usually abolishes Meiotic sex chromosome inactivation (MSCI) and
246 increases the expression of X-linked and Y-linked genes in spermatocyte (25). To evaluate
247 whether the deficiency of *circSry* impacted MSCI, we calculated the average value of gene
248 expression from individual chromosomes based on our RNA-seq analysis in *circSry* KO versus
249 control. The average value of all autosomal genes expression was 1.04 (Figure 4 figure
250 supplement 3D), showed that the genes expression level of all autosome chromosomes from
251 *circSry* KO spermatocytes was not different from control. However, the average values of X and
252 Y-linked genes were 1.93 and 2.15, respectively (Figure 4 figure supplement 3D). These data

253 indicated that *circSry* deficiency resulted in the failure of inactivating some of the sex
254 chromosome-linked genes in *circSry* KO spermatocytes. In particular, X-linked genes *Rbbp7*,
255 *Pgk1* (27, 28), and Y-linked gene *Eif2s3y*, known to be subjected to MSCI (25, 29), were up
256 regulated in *circSry* KO spermatocytes, whereas the expression of autosome genes *Xpo6* and
257 *Dhrs1* were not affected (Figure 4 figure supplement 3E). Taken together, we conclude that the
258 loss of *circSry* impairs MSCI.



259
260 **Figure 4. Loss of circSry leads to decreased number and meiotic arrest in primary spermatocytes.** (A)
261 Immunofluorescence staining of SYCP3 in seminiferous tubules of *circSry* KO (down) and control mice (up). Scale bars
262 indicate 50μm. (B) Quantification of SYCP3 positive cells within seminiferous tubules from *circSry* KO compared with

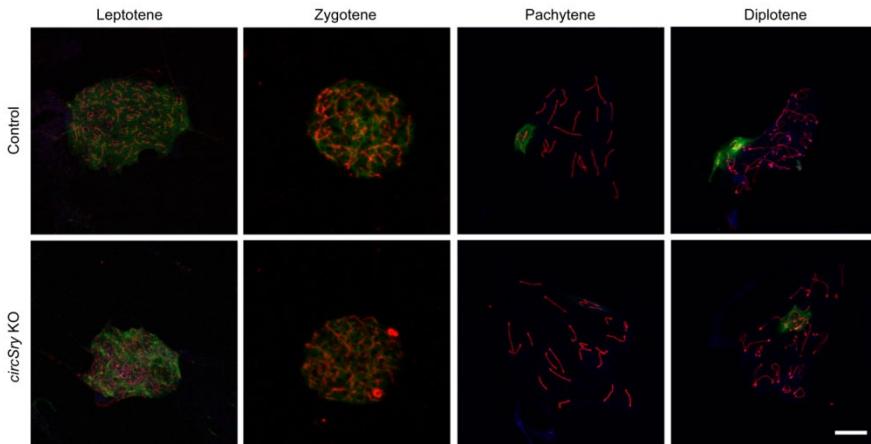
263 control mice (****p< 0.0001; unpaired, two tailed t test, n=70). (C) Flow cytometry analysis of proportion of 4n cells, 1n
 264 cells and 2n cells in 8-weeks mice testes between control and *circSry* KO mice(n=3). (D) (a to h)Representative image of
 265 Immunofluorescence co-staining of Tunel signal (green) and SYCP3 (red) in 9-week old control or *circSry* KO mice
 266 testes.(a to f Scale bars indicate 50 μ m; g, h Scale bars indicate 25 μ m).(E) Expression of H2AX mRNA in control and
 267 *circSry* KO mice spermatocytes (**p<0.01; unpaired, two tailed t test, n=5) (F) Representative image of western blot
 268 results of γ H2AX in control and *circSry* KO mice spermatocytes (n=3). (G and H) Immunofluorescence staining of SYCP3
 269 (red) and γ H2AX (green) or SYCP1 (green) in control and *circSry* KO spermatocytes at early pachytene stage. Arrowhead
 270 indicates the Y chromosome; arrow indicates the X chromosome. Scale bars indicate 100 μ m. (I)The proportion of
 271 leptotene, zygotene, pachytene and diplotene spermatocytes from control or *circSry* KO mice testes .The data are
 272 presented as the mean \pm s.e.m.



273

274 **Figure 4 figure supplement 2. RNA-seq analysis between *circSry* KO and control primary spermatocytes (related**
 275 **to Figure 4).** (A) Heat map illustrates RNA-seq differential expression data. Red, positive fold-change (logFC) indicates
 276 higher expression; blue, negative logFC. Total RNA was extracted from primary spermatocytes of control or *circSry* KO
 277 mice. (B) Enrichment analysis of significant down regulation comparing with control mice, FDR<0.05, logFC<-1. (C) H2AX

278 expression comparing with control and *circSry* KO spermatocytes (*p<0.05, t test, n=3). (D) Relative gene expression
279 levels on chromosomes as determined by RNA-seq (n=3). (E) Relative expression of X-linked genes, *Rbby7*, *Pgk1*; Y-
280 linked genes, *Eif2s3y*; and genes on autosome, *Xpo6*, *Dhrs1*, as determined by RNA-seq analysis on control and *circSry*
281 KO spermatocytes (**p<0.01, *p<0.05, ns, not significant; n=3). The data are presented as the mean \pm s.e.m.



282
283

284 **Figure 4 figure supplement 3. Staining SYCP3 (red) and γH2AX (green) in control and *circSry* KO spermatocytes**
285 from leptotene to diplotene stages (related to Figure 4). (Up) Representative image of immunofluorescence staining of
286 SYCP3 (red), γH2AX (green) and DAPI (blue) in 8-week old control or *circSry* KO mice testes (Down). Scale bars indicate
287 10 μ m. (Two tailed t test, ***p<0.001, ns, not significant). Total 400 cells (300 cells of pachytene stage, 100 cells of the
288 other stages) were counted from 3 mice.

289 ***CircSry* regulates γH2AX expression via sponging miR138-5p**

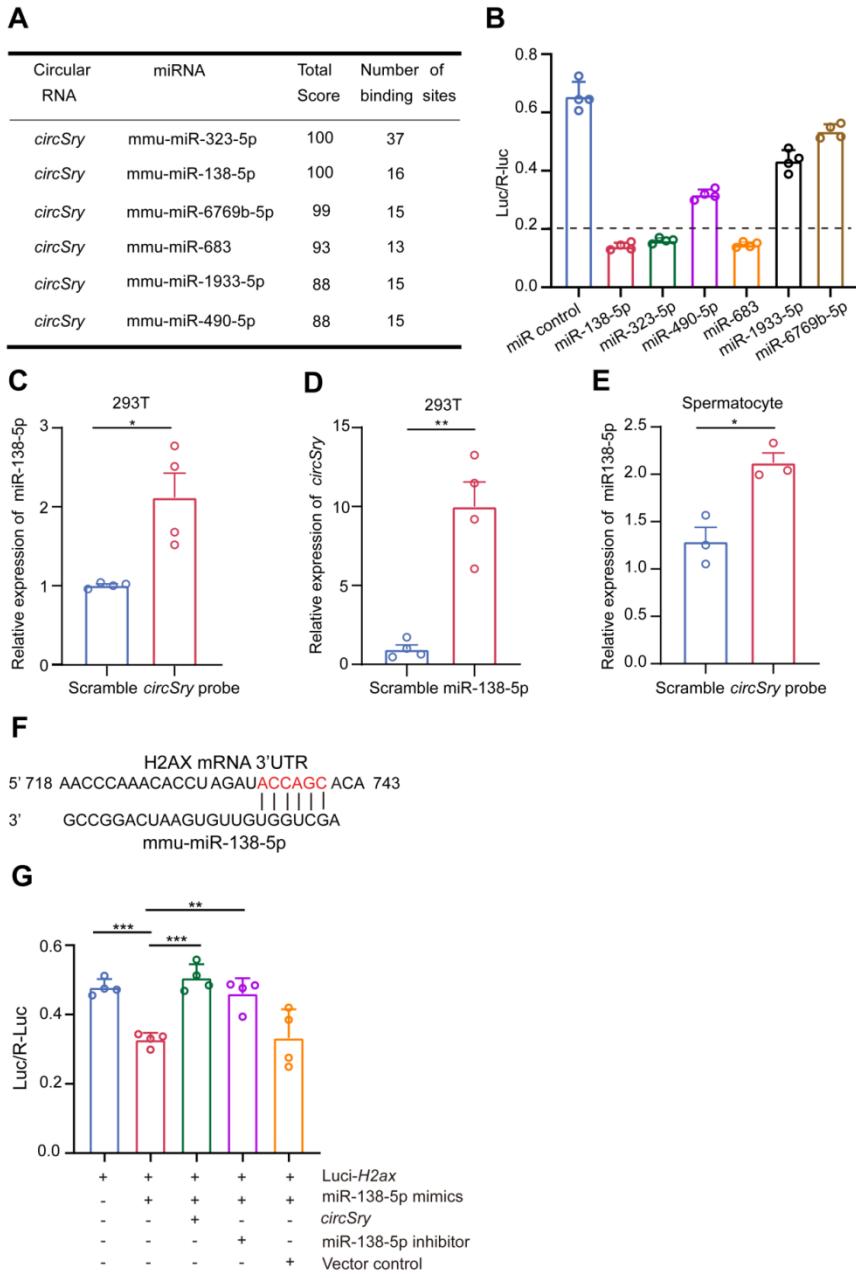
290 Since cytoplasmic circRNAs could act as miRNA sponge to regulate gene expression indirectly,
291 we predicted potential miRNAs that interacted with *circSry* using web tool miRDB (30, 31). 33
292 miRNAs were predicted to potentially interact with *circSry*, 6 of which had more than 7 binding
293 sites on *circSry*, including well characterized miR-138-5p (Figure 5A and Table S1) (13). To
294 validate these miRNAs using luciferase assay, we constructed reporter plasmid by inserting
295 *circSry* sequence into the 3'UTR of luciferase coding sequence. Compared with scramble miRNA,
296 miR-138-5p, miR-323-5p, and miR-683 reduced the luciferase signals to the greatest extent
297 (Figure 5B). Furthermore, among these miRNAs, miR-138-5p was the most abundantly
298 expressed in mouse male germ cells based on published miRNA sequencing data (32, 33).
299 Therefore, we hypothesized that *circSry* regulated histone H2AX by sequestering miR-138-5p.

300 To test whether *circSry* binds AGO2-miR-138-5p complex, we performed AGO2
301 immunoprecipitation on isolated spermatocytes. *CircSry* was specifically enriched in AGO2
302 immunoprecipitates (Figure 5 figure supplement 1A), indicating that *circSry* interacts with miRNAs.
303 Next, we performed RNA-RNA pull down experiment to examine the interaction between miR-
304 138-5p and *circSry* in 293T cell line that highly expressed *circSry* (Figure 5 figure supplement 1B).
305 This cell line was established by transducing a lentiviral vector harboring the *circSry* expressing
306 cassette. The splicing site of *circSry* was also confirmed by Sanger sequencing (Figure 5 figure
307 supplement 1C). Pull down experiment showed that *circSry* biotinylated probe significantly
308 enriched the miR138-5p compared to the scramble probe (Figure 5C), and biotin-coupled miR-
309 138-5p captured more *circSry* than the biotin control miRNA (Figure 5D). Furthermore, RNA-RNA
310 pull down in spermatocytes showed that *circSry* probe captured more miR-138-5p than scramble
311 probe as well (Figure 5E). Taken together, these results suggest that *circSry* acts as a sponge for
312 miR-138-5p.

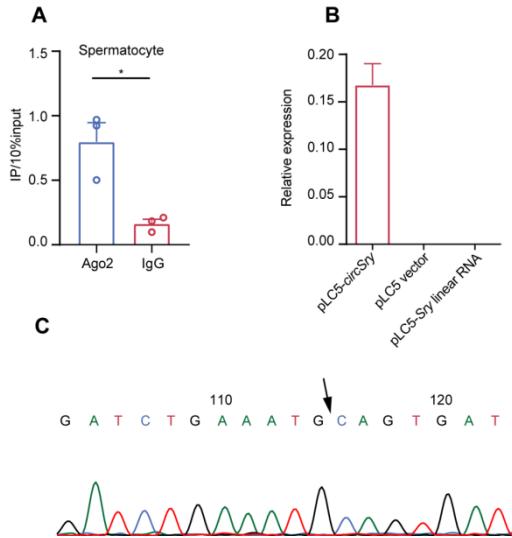
313 It has been reported that miR-138-5p directly down regulates H2AX expression through
314 binding to 3'UTR region of H2AX mRNA (Figure 5F), inducing chromosomal instability during
315 DNA damage repair (34). To test our hypothesis that *circSry* regulates H2AX expression via
316 sponging miR-138-5p during spermatogenesis, we conducted luciferase reporter experiments.
317 Transfection of luciferase reporter containing H2AX sequence together with miR-138-5p mimics
318 showed significantly reduced luciferase signal, while co-transfection of *circSry* rescued the
319 decrease of luciferase signal (Figure 5G).

320 Taken together, these results show that *circSry* regulates the expression of H2AX mRNA at
321 post-transcriptional level by sponging miR-138-5p and contributes to MSCI during
322 spermatogenesis.

323



324
325 **Figure 5. CircSry enhances γH2AX expression by sponging miR138-5p in spermatocytes.** (A) Table of 6 miRNAs
326 that have more than 7 potential binding sites for *circSry*. (B) Luciferase reporter assay showing diverse binding capacities
327 of 6 miRNAs on Luc-Sry (n=4) (C) RNA-RNA pull down enrichment of miR-138-5p using *circSry* biotinylated probe or
328 scramble probe in 293T cell line .(*p<0.05;unpaired, two tailed t test, n=4).(D) RNA-RNA pull down enrichment of *circSry*
329 using biotin-coupled miR-138-5p in 293T cell line .(**p<0.01; unpaired, two tailed t test, n=4) . (E) Expression level of
330 miR-138-5p pulled down by *circSry* biotinylated probe or scramble probe in spermatocytes (*p < 0.05, unpaired, two tailed
331 t test n=4). (F) A putative binding site between miR-138-5p and H2AX mRNA 3'UTR. (G) Luciferase reporter assay of
332 *circSry* on sponging miR-138-5p. MiR-138-5p inhibitor and empty vector were used as positive and negative control,
333 separately (**p<0.01, ***p<0.001; unpaired, two tailed t test; n=4). The data are presented as the mean ± s.e.m.



334
335

336 **Figure 5 figure supplement 1. Overexpression of circSry in 293T cell line (related to Figure 5).** (A) RIP results
337 showing the *circSry* enrichment using AGO2 antibody vs IgG antibody *in vivo* (* $p<0.05$; unpaired, two tailed t test, n=3).
338 (B) Relative expression of *circSry* and *Sry* linear RNA were measured with 293T cells line that expressed *circSry*. (C)
339 Sanger sequencing showed junction sequence of *circSry* (Arrow indicates the junction site). The data are presented as
340 the mean \pm s.e.m.

341

342 Discussion

343

344 Recent studies have identified thousands of circular RNAs and many of them have important
345 biological functions in various tissues and organs. Here we show that a circular transcript exhibits
346 regulatory function in male reproductive system. Even more interesting, this circular RNA
347 originates from the sex-determining gene *Sry*, which initiates the male germ cell development in
348 the first place. In this study, we show that loss of *circSry* led to defective spermatogenesis,
349 specifically causing primary spermatocytes apoptosis. Deletion of *circSry* decreased the
350 expression of H2AX and displayed aberrant XY synapsis at pachytene stage, followed by MSCI
351 abolishment. Given that miR-138-5p directly targets H2AX mRNA (34), we performed RNA-RNA
352 pull down experiment and luciferase reporter assay, demonstrating that *circSry* enhanced H2AX
353 expression by sequestering miR-138-5p.

354 Mouse *Sry* gene is capable of producing linear RNAs at embryonic stage as well as circular
355 transcripts in adult testis. It was proposed that *circSry* is generated from a linear RNA precursor
356 containing long palindromic repeats, which is transcribed from a distal promoter (35). How does
357 the transcription of *Sry* differentially regulated in embryonic genital ridge and adult male germs
358 cells is poorly understood. This mechanism of alternative promoter choice and upstream
359 regulation is an interesting direction of future study.

360 In addition to *Mus musculus*, transcripts of *Sry* were also detected in the testes of *Mus*
361 *musculus domesticus* and *Mus spretus*. Considering that splicing donor site was also conserved
362 in *Rattus norvegicus* (21), we speculated that alternative splicing occurred in rat as well. Do these
363 transcripts form circular RNA, and do they regulate spermatogenesis need to be further explored.
364 *CircSry* was also expressed in the mouse brain during embryonic stage and its expression was
365 diminished after birth (36). Whether *circSry* plays a role in mouse brain needs further
366 investigation.

367 Human *SRY* is also expressed in adult testis. Different from the circular transcript in adult
368 mouse testis, however, human *SRY* transcript is liner and polyadenylated (37), and the presence
369 of circular *SRY* has not been reported. The function of human *SRY* transcript in adult testes is
370 very curious and the elucidation of which may help us to better understand male development
371 and spermatogenesis in human. While human *SRY* transcript in testes might regulate germ cell
372 development using a different mechanism, the sex determining gene also plays a role in adult
373 testes could be a common theme in multiple mammalian species.

374 Taken together, our study complements *Sry*'s role in germ cell development, revealing its
375 significance in male germ cell development. *CircSry* safeguards the inactivation of sex
376 chromosomes during pachytene stage, the key process to complete meiosis and produce
377 sperms. Upon fertilization, the sperm carrying Y chromosome contributes Y to the embryo which
378 will develop into a male and grow testicles. The cycle of *Sry* expression and duel forms and
379 functions suggests a mechanism to ensure the preservation of vulnerable Y chromosome in
380 evolution.

381

382 **Materials and Methods**

383

384 **Animals**

385 The mice used in this research were C57BL/6 background. All animals were maintained at 24°C
386 and 50–60%humidity under a 12:12 h light/dark cycles and with ad libitum access to food and
387 water. *Sry*^{fl}_{ox} mice were generated in Jackson Laboratory. *Stra8-Cre* and *Amh-Cre* mice were
388 provided by Prof. Gao of Institute of Zoology, University of Chinese Academy of Sciences. All
389 mice studies were carried out in accordance with the principles approved by the Institutional
390 Animal Care and Use Committee at the Institute of Zoology, Chinese Academy of Sciences.

391 **Establishment of mutant mice by CRISPR/Cas9-based genome editing**

392 *CircSry* knockout mice were established by microinjection of Cas9 mRNA and single-guide RNAs
393 (sgRNAs) into zygotes (38). All RNAs prepared for microinjection were in vitro transcribed. Briefly,
394 Cas9 mRNAs and sgRNAs were mixed properly and injected into zygotes. The injected embryos
395 were transferred to pseudopregnant females. To genotype mutant mouse lines, genomic
396 sequence was amplified by PCR, followed by Sanger sequencing. All oligonucleotides were listed
397 in the Table S2.

398 **Establishment of *Sry*^{fl}_{ox} mice by CRISPR/Cas9 system**

399 We used single-stranded oligodeoxynucleotides (ssODN) to establish knock-in mouse mutants
400 (38). Establishment of *Sry*^{fl}_{ox} KI mice went through two rounds of microinjections. The first
401 microinjection contained Cas9 mRNA and ssODN (V5-loxP) targeted the 3' end of *Sry* exon 1 (V5-
402 TGA-loxP). The injected embryos were transferred to pseudopregnant females. The correct male
403 mouse line was determined by Sanger sequencing of genomic sequence. Sperms from *Sry*-V5-
404 loxP male mouse line were collected for single-sperm microinjection of wild-type oocytes. The
405 second microinjection contained Cas9 mRNA and ssODN (5'-loxP) targeted the 5' upstream site
406 of *Sry* exon1 start codon within the *Sry*-V5-loxP zygotes. The correct mouse line was again

407 confirmed by Sanger sequencing of genomic sequence. All oligonucleotides and primers
408 sequences were listed in the Table S2.

409 **Genotyping of mice and sexing**

410 All mice were genotyped with the tail DNA. Chromosomal sex was detected through amplifying
411 the Y-linked gene *Uty*. Phenotypic sex was determined by examination of external genitalia, and
412 the presence /absence of mammary glands. Primers were listed in Table S2.

413 **RNA preparation and real-time PCR**

414 Nuclear and cytoplasmic RNAs were extracted by using Norgen's Cytoplasmic & Nuclear RNA
415 Purification Kit (Norgen Bitek Corp.). RNAs were extracted using Trizol reagent (Life
416 Technologies). cDNAs were synthesized using the Hifair III 1st strand cDNA synthesis reagent
417 (Yeasen company, China) with 500ng of total RNA. cDNAs were amplified with Hieff qPCR SYBR
418 Green Master mix (Yeasen company, China) and quantified with Roche LightCycle 480 system.
419 For RNase R treatment, experiment was performed by incubation of 3 µg of RNA with 6U µg⁻¹ of
420 RNase R (Epicenter) for 25 minutes at 37°C. The expression levels of each gene were presented
421 relative to GAPDH or U6 expression. The primers sequences were listed in Table S2.

422 **RNA pull-down**

423 Biotinylated *circSry* probe and 5'bio-miRNA mimic were synthesized by RiboBio (Guangzhou,
424 China). Testes were ground and incubated in lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl,
425 2 mM MgCl₂, 1% NP40, RNase Inhibitor (Beyotime biotechnology)] on ice for 1 hour. The lysates
426 were then incubated with the biotinylated probes at RT for 4 hours; followed by adding
427 streptavidin C1 magnetic beads (Invitrogen) were to binding reaction, and continued to incubate
428 at 4°C , overnight. On second day, the beads were washed briefly with wash buffer [0.1% SDS, 1%
429 Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, and 500 mM NaCl] five times. The bound RNA in the

430 pull-down was further extracted for purification and RT-qPCR. The sequence of *circSry* probe
431 was shown in Table S3.

432 **RNA-binding protein immunoprecipitation**

433 RIP experiments were performed with primary spermatocytes which separated from adult mice
434 testes and homogenized into a single-cell suspension in ice-cold PBS. After centrifugation, the
435 pellet was resuspended in RIP lysis buffer. Magnetic beads were incubated with 5 μ g antibody
436 against AGO2 (Proteintech Inc), or IgG at RT. The tissue lysates were then incubated with the
437 bead-antibody complexes overnight at 4°C. RNAs were extracted by Trizol reagent and reverse-
438 transcribed after proteinase K treatment.

439 **RNA-seq and data analysis**

440 RNA sequencing reads were aligned to mouse reference sequence GRCm39 using STAR
441 (2.7.0f). Read counts and TPM (Transcripts Per Kilobase Million) were counted using RSEM
442 (1.3.2). Differential expression genes (DEG) (FDR < 0.05, log2 (fold change) (log2 FC) \geq 1 or \leq
443 -1) were calculated by edgeR (3.28.1) package. The DEG genes were carried out Gene
444 Ontology Pathway analysis and Kyoto Encyclopedia of Genes and Genomes analysis by
445 clusterProfiler (3.14.3) and org.Hs.eg.db (3.10.0) packages. Heatmap (1.0.12) was used for the
446 heatmap visualization; colors represent the Z-score derived from the log2 transformed FPKM
447 data. Principal Component Analysis (PCA) was performed by R packages, including tidyR (1.1.2),
448 dplyr (1.0.2) and ggplot2 (3.3.2).

449 **Construction of *circSry* expression vector**

450 The empty vector was purchased from Genesee Bio (Guangzhou,China), termed pLC5. This
451 plasmid was linearized with Xhol-BamHI. Complete *circSry* sequence was amplified with Xhol-
452 BamHI clone site and inserted into pLC5. The final plasmid was named pLC5-*circSry*. After
453 sequencing, pLC5-*circSry* was transfected into 293T cell line to testify the expression of *circSry*.

454 **Dual-luciferase reporter assay**

455 The full-length sequence of *circSry* or the 3'UTR of H2AX was inserted into the 3' UTR of pMIR-
456 Report Luciferase vector (gifted by Yu Wang lab, State Key Laboratory of Stem Cell and
457 Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing). Co-
458 transfection of 500 ng pMIR-Report luciferase vector, miRNA mimic (RiboBio) and pLC5-*circSry*
459 were conducted using lipofectamine 2000 (Invitrogen). After 48 hours, the luciferase activities
460 were measured using a dual-luciferase reporter assay kit (Promega). The results were
461 normalized to the ratio between Firefly signal and Renilla signal.

462 **Western blotting**

463 Protein obtained from spermatocytes were separated by gel electrophoresis SDS-PAGE and
464 transferred to a PVDF membrane. The PVDF membrane was incubated with primary antibodies
465 against γ H2AX (Millipore; 05-636, 1:200), β tubulin (Yeasen company, China; 1:1000) 4°C
466 overnight and horseradish peroxidase-labeled secondary antibody for 1 hour at 37°C
467 subsequently. Images were captured using ECL Western Blotting Substrate (Thermo Scientific
468 Pierce).

469 **Tunel assays**

470 The TUNEL assay was performed by TUNEL BrightRed Apoptosis Detection Kit (Vazyme). Briefly;
471 sections were permeabilized by protein K and labeled with rTdT reaction mix for 1 hour at 37°C.
472 Reaction was stopped by 1× PBS. After washing in PBS, the sections were incubated in 2 μ g/ml
473 of DAPI (Molecular Probes, D1306) for 10 minutes. Sections were mounted on slides with Dako
474 Fluorescence Mounting Medium (Dako Canada, ON, Canada). Images were obtained using a
475 laser scanning confocal microscope LSM780 (Carl Zeiss).

476 **Flow cytometry**

477 The cell suspensions obtained from testes were digested by collagenase IV and Typsin for 5
478 minutes (39). To analyse DNA ploidy, the cells were incubated with the Hoechst for 15 minutes
479 and filtered before being subjected to flow cytometry. The results were analysed using a FACS-
480 Calibur system (BD Biosciences).

481 **Sperm count and fertility**

482 The epididymis tail of the mouse was taken out, cut into pieces with ophthalmic scissors, placed
483 in a 37°C water bath for 15 minutes, and counted via microscope. 2-3-month-old *circSry* and
484 control C57BL/6J male mice were housed with control C57BL/6J males (2–3-month-old), which
485 were proved having normal fecundity. Copulatory plugs were monitored daily, and plugged
486 females with visibly growing abdomen were moved to separate cages for monitoring pregnancy.
487 The mating process lasted for 4 months. The numbers of pups (both alive and dead) were
488 counted on the first day of life.

489 **Tissue collection and histological analysis**

490 Testes from control and *circSry* KO mouse were dissected immediately after euthanasia, then
491 immediately fixed in 4% paraformaldehyde (PFA) for 24 hours, after storing in 70% ethanol and
492 embedding in paraffin, 5 µm-thick sections were prepared using a rotary microtome (Leica) and
493 mounted on glass slides. Sections were stained with MVH (Abcam) for histological analysis.

494 **Immunofluorescence analysis**

495 After deparaffinization and antigen retrieval, 5% bovine serum was used to block sections at room
496 temperature (RT) for 1 hour, and specific primary antibody was used to incubate with sections at
497 RT or overnight at 4°C. After washing the sections for three times, the slides were incubated with
498 the corresponding secondary antibody, fluorescent dye-conjugated-FITC or TRITC (1:150,
499 Jackson) for 1 hour at RT (Avoiding the light). DAPI was used to stain the nucleus. All images
500 were captured with a confocal microscopy (Leica TCS SP8). All the antibodies were listed in the
501 methods and materials.

502 **Preparation of synaptonemal complex**

503 Seminiferous tubules were collected from dissecting testes and washed with 1× PBS. Hypo
504 extraction buffer (HEB) was used to incubate within seminiferous tubules for 30 minutes, followed
505 by disrupting within 0.1 M sucrose liquid to form a single-cell suspension. The cell suspension
506 was mounted on slides treated with 1% PFA. Slides were air-dried in a humidified box for at least
507 6 hours. After washing with 0.04% Photo-Flo (Equil, 1464510), the slides were staining for SYCP3.
508 Incubating with SYCP3 antibody was conducted at RT for 30 minutes after antibody dilution buffer
509 (ADB) treatment. After washing three times in 1× Tris buffer, saline (TBS), blocking was
510 conducted with 1× ADB at 4°C overnight. After washing three times with cold 1× TBS buffer,
511 corresponding secondary antibody, and fluorescent dye-conjugated-TRITC were incubated with
512 sections for 3 hours at 37°C. All images were captured with a confocal laser scanning microscope
513 (Leica TCS SP8).

514 **Data and statistical analysis**

515 All images were processed with Photoshop CS6 (Adobe). All statistics were analyzed using Prism
516 software (GraphPad Software). All experiments were confirmed with at least three independent
517 experiments, and three to five control or mutant testes were used for immunostaining. The
518 quantitative results were presented as the mean \pm s.e.m. The significant difference was evaluated
519 with t-test. P-value < 0.05 was considered as significant.

520 **Data availability**

521 RNA sequencing-derived data reported in this study have been deposited in NCBI's Gene Expression
522 Omnibus (GEO) under accession number GSE185184. Reviewers go to GEO with the link:
523 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE185184>. A private token is provided for reviewers
524 to check the records: sditsmkadvqvfqh.
525 Source data is provided as Supplementary file2.

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534

535 **Competing Interest Statement:** The authors declare no competing interests.

536

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618

620 **Table S1. Predicted miRNAs that bind *circSry***

Circ Name	miRNA	Score	Binding Sites count	Positions
<i>circSry</i>	mmu-miR-323-5p	100	37	573, 576, 602, 606, 653, 657, 683, 687, 713, 717, 720, 723, 726, 749, 753, 779, 824, 828, 854, 858, 861, 864, 890, 920, 950, 980, 1010, 1014, 1017, 1049, 1053, 1079, 1083, 1109, 1113, 1139, 1143
<i>circSry</i>	mmu-miR-138-5p	100	16	483, 578, 608, 659, 689, 755, 782, 830, 893, 923, 1019, 1055, 1085, 1115, 1145, 1194
<i>circSry</i>	mmu-miR-6769b-5p	99	15	577, 607, 658, 688, 727, 754, 781, 829, 892, 922, 1018, 1054, 1084, 1114, 1144
<i>circSry</i>	mmu-miR-3098-3p	97	7	176, 662, 833, 959, 1028, 1058, 1088
<i>circSry</i>	mmu-miR-683	93	13	176, 589, 662, 700, 766, 833, 871, 959, 997, 1028, 1058, 1088, 1126
<i>circSry</i>	mmu-miR-485-5p	86	1	326
<i>circSry</i>	mmu-miR-1962	89	1	326
<i>circSry</i>	mmu-miR-1933-5p	88	15	649, 709, 745, 775, 820, 850, 886, 916, 946, 976, 1006, 1045, 1075, 1105, 1135
<i>circSry</i>	mmu-miR-490-5p	88	15	648, 708, 744, 774, 819, 849, 885, 915, 945, 975, 1005, 1044, 1074, 1104, 1134
<i>circSry</i>	mmu-miR-1903	87	1	59
<i>circSry</i>	mmu-miR-12183-5p	87	1	59
<i>circSry</i>	mmu-miR-6908-5p	85	1	441
<i>circSry</i>	mmu-miR-7063-3p	82	1	97
<i>circSry</i>	mmu-miR-6973b-3p	82	1	97
<i>circSry</i>	mmu-miR-1904	81	7	177, 663, 834, 960, 1029, 1059, 1089
<i>circSry</i>	mmu-miR-7021-3p	80	4	200, 276, 296, 351
<i>circSry</i>	mmu-miR-93-3p	77	7	176, 662, 833, 959, 1028, 1058, 1088
<i>circSry</i>	mmu-miR-7011-3p	75	6	667, 838, 964, 1033, 1063, 1093
<i>circSry</i>	mmu-miR-3078-3p	75	5	174, 185, 484, 957, 987
<i>circSry</i>	mmu-let-7a-2-3p	73	1	395
<i>circSry</i>	mmu-miR-7035-5p	70	4	174, 184, 957, 987
<i>circSry</i>	mmu-miR-7662-5p	70	1	26
<i>circSry</i>	mmu-miR-7079-5p	70	1	26
<i>circSry</i>	mmu-miR-7028-5p	70	1	26
<i>circSry</i>	mmu-miR-6911-5p	70	1	26
<i>circSry</i>	mmu-miR-367-5p	67	5	586, 697, 763, 994, 1123
<i>circSry</i>	mmu-miR-103-2-5p	61	6	666, 837, 963, 1032, 1062, 1092
<i>circSry</i>	mmu-miR-103-1-5p	61	6	666, 837, 963, 1032, 1062, 1092
<i>circSry</i>	mmu-miR-107-5p	56	6	666, 837, 963, 1032, 1062, 1092

<i>circSry</i>	mmu-miR-3970	56	1	399
<i>circSry</i>	mmu-miR-12184-5p	54	1	190
<i>circSry</i>	mmu-miR-7656-3p	50	2	371, 503
<i>circSry</i>	mmu-miR-6896-3p	50	1	300

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