

1 **Identification and characterization of BEND2 as a novel and key**
2 **regulator of meiosis during mouse spermatogenesis**

3 Longfei Ma^{1,2*}, Dan Xie^{1,2,3*}, Xiwen Lin^{1,2}, Hengyu Nie^{1,2,3}, Jian Chen^{1,2}, Chenxu Gao³,
4 Shuguang Duo^{3†}, Chunsheng Han^{1,2,3†}

5 ¹State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology,
6 Chinese Academy of Sciences, Beijing 100101, China

7 ²Innovation Academy for Stem Cell and Regeneration, Chinese Academy of Sciences,
8 Beijing 100101, China

9 ³Institute of Zoology, University of Chinese Academy of Sciences, Chinese Academy
10 of Sciences, Beijing 100049, China

11 *These authors contributed equally to this work.

12 Correspondence authors: hancs@ioz.ac.cn, duoshuguang@ioz.ac.cn.

13

14 **Abstract**

15 The chromatin state undergoes global and dynamic changes during spermatogenesis,
16 and is critical to chromosomal synapsis, meiotic recombination, and transcriptional
17 regulation. However, the key regulators involved and the underlying molecular
18 mechanisms remain poorly understood. Herein we report that mouse BEND2, one of
19 the BEN-domain- containing proteins conserved in vertebrates, was specifically
20 expressed in spermatogenic cells within a short time-window spanning meiotic
21 initiation, and that it plays an essential role in the progression of prophase in meiosis
22 I. *Bend2* gene knockout in male mice arrested meiosis at the transition from
23 zygonema to pachynema, disrupted synapsis and DNA double-strand break repair, and
24 induced non-homologous chromosomal pairing. BEND2 interacted with a number of
25 chromatin-associated proteins—including ZMYM2, LSD1, CHD4, and ADNP—

26 which are components of certain transcription-repressor complexes. BEND2-binding
27 sites were identified in diverse chromatin states and enriched in simple sequence
28 repeats. BEND2 contributed to shutting down the mitotic gene-expression program
29 and to the activation of meiotic and post-meiotic gene expression, and it regulated
30 chromatin accessibility as well as the modification of H3K4me3. Therefore, our study
31 identified BEND2 as a novel and key regulator of meiosis, gene expression, and
32 chromatin state during mouse spermatogenesis.

33 **Teaser**

34 Meiosis is a highly complex yet poorly understood process that involves the concerted
35 actions of an increasing number of regulators, of which the list remains incomplete.
36 Ma et al. identified BEND2 as a novel and key regulator of meiosis and showed that it
37 interacts with critical chromatin modulators and specific genomic elements to control
38 the expression of mitotic and meiotic genes.

39 **INTRODUCTION**

40 Meiosis is the fundamental component of gametogenesis and consists of multiple
41 processes that occur either sequentially or concurrently (1, 2). Meiosis is initiated
42 when homologous chromosomes begin to pair and large-scale, programmed DNA
43 double-strand breaks (DSBs) are generated (3). DSB repair and synapsis of
44 homologous chromosomes are simultaneous and mutually dependent. Synapsis starts
45 when the 3' overhangs of DSBs invade homologous DNAs to form recombinant
46 intermediates, and when the axial elements of the synaptonemal complex that consists
47 of proteins such as SYCP3 and cohesin are bridged by SYCP1 and other central
48 element components (4). Full synapsis is achieved when DSB repair intermediates are
49 resolved into crossovers and the chromosomes become highly condensed around the
50 complete synaptonemal complex.

51 These meiotic steps/processes are intricately coordinated by the complex
52 interactions between chromatin and a large number of chromatin-binding proteins that
53 include synaptonemal complex proteins, enzymes, chromatin modifiers/remodelers,
54 and transcription factors (5). To give an example, PRDM9 acts as both a histone-

55 modifying enzyme and a pioneer transcription factor, and interacts either directly or
56 indirectly with many proteins—including CXXC finger protein 1 (CXXC1), EWS
57 RNA binding-protein 1 (EWSR1), euchromatic histone lysine methyltransferase 2
58 (EHMT2), chromodomain Y like (CDYL), meiotic cohesin REC8, SYCP3, SYCP1,
59 and lymphoid-specific helicase (LSH/HELLS) (6, 7). An increasing number of
60 meiotic regulators have been identified by genetic studies, using model organisms
61 such as gene knockout (KO) mice; these meiotic regulators include chromatin
62 remodelers/modifiers and transcription factors such as HELLS (8, 9), YY1 (10), TET1
63 (11), INO80 (12), BRG1(13), Suv39h (14), DNMT3L (15), EHMT2 (16), PRDM9
64 (17), MLL2 (18), and SCML2 (19). Unfortunately, the spatiotemporal interactions
65 between these regulators remain largely unknown. The concerted actions of these
66 regulators usually result in chromatin states that are required for DNA activities such
67 as DSB formation/repair, synapsis, and transcription (20-24). Specifically, correct
68 chromatin states at particular genomic regions such as repetitive sequences and
69 heterochromatin must be established to prevent erroneous recombination and/or
70 transcription, which are detrimental to genome integrity (10, 14).

71 In the present study, we report the identification of a novel meiotic regulator,
72 BEND2, that belongs to a BEN-domain-containing protein family that is poorly
73 characterized. The BEN domain was first identified in diverse metazoan and viral
74 proteins (usually with multiple copies), and was named after three experimentally
75 characterized proteins—BANP, E5R, and NAC1—in which it is present (25). A total
76 of nine human and mouse genes that encode BEND1-9 are found in each of the
77 genomes of these two species according to the NCBI Gene database. Although studies
78 on the BEN family members are limited, they reveal the following key points: 1) BEN
79 proteins tend to interact with a variety of proteins, most likely in a context-dependent
80 manner; 2) most of the interacting proteins are components of transcription-repressive
81 complexes involved in chromatin remodeling and/or modification; and 3) BEN
82 proteins can be sequence-specific DNA-binding proteins (see Discussion for more
83 details).

84 To our knowledge, there is no report regarding the functions of BEN proteins in
85 germ cell development. In the present study, we showed that BEND2 is specifically
86 expressed in spermatogenic cells shortly before and during prophase of meiosis I, and

87 is essential for meiosis in male mice. We observed multiple meiotic defects in DSB
88 repair and synapsis in male KO mice, such as complete spermatogenic arrest at
89 zygonema. We also demonstrated that BEND2 interacts with multiple chromatin-
90 binding proteins, and that it regulates chromatin states and transcription by
91 preferentially targeting simple sequence repeats. These results add to our
92 understanding of the molecular mechanisms governing meiosis and cell-specific
93 regulation of chromatin states in meiotic cells.

94 **RESULTS**

95 **BEND2 is a novel protein that is specifically expressed in spermatogenic cells** 96 **around the time of meiotic initiation**

97 We were initially interested in identifying and analyzing the functions of long
98 noncoding RNA (lncRNA) genes that are specifically expressed in spermatogenic
99 cells. An X chromosome-linked lncRNA gene based on the NCBI gene annotation
100 was one such candidate, as we found that its transcripts were specifically expressed in
101 mouse testes based upon our RNA-seq analyses of mouse multi-organ transcriptomic
102 data (26). While our study was ongoing, this gene was re-annotated as encoding a
103 protein belonging to the BEN family (25) (Fig. 1A). As the orthologous protein in
104 humans has been named BEND2, we suggest that this mouse protein also adopt the
105 same name. Predicted homologous BEND2 proteins can be found in vertebrates from
106 fish to humans, and the sequence identity between the mouse and human proteins is
107 34% (Fig. S1A, B). The predicted longest transcript of mouse *Bend2* contains 15
108 exons, of which exons 2–15 harbor a coding sequence (CDS) for a protein of 728 aa
109 (predicted molecular mass, 80 kDa) (Fig. 1A). And these transcripts were indeed
110 detected exclusively in mouse testes by RT-PCR (Fig. 1B, S1C).

111 We developed a rabbit polyclonal antibody to BEND2 (rpAb-B2) by using a 30-
112 aa synthetic polypeptide located between the two BEN domains (Fig. S1B). This
113 antibody functioned appropriately in western immunoblotting and
114 immunohistochemical analyses (Fig. S1D–F). By using rpAb-B2 in western blotting
115 assays, we detected two proteins related to BEND2 in the testes of WT but not *Bend2*
116 KO mice: one was 140 kDa (p140), while the other was 80 kDa (p80) (Fig. S1D).

117 Intriguingly, p80 but not p140 could be consistently and specifically found in testes
118 (Fig. S1E). As a non-specific protein was also detected by rpAb-B2 upon western blot
119 analysis (labeled with an asterisk in Fig. S1D), we decided to use CRISPR-Cas9
120 technology to generate knock-in (KI) mice in which a 3XFLAG sequence was added
121 to the N-terminus of BEND2 (FLAG-BEND2) (Fig. 1C, S1G). By using a mouse
122 monoclonal Ab against FLAG (mmAb-FLAG), p140 but not p80 was detectable in the
123 KI testes but not in the WT or the KO testes by westerns (Fig. S1H). p140 was also
124 specifically observed when FLAG-BEND2 cDNA was expressed in 293FT cells by
125 both rpAb-B2 and mmAb-FLAG, suggesting that p140 protein was FLAG-BEND2
126 itself (Fig. S1I, J). Based on these results, it is likely that p140 is the full-length
127 BEND2, the mobility of which using SDS-PAGE was altered due to either post-
128 translational modification(s) or unusual higher-order structures; p80 was a shorter
129 version of BEND2, likely due to either alternative transcription or translation, or
130 protein cleavage from p140. We next expressed the N- and C-terminal halves of
131 BEND2 as FLAG-tagged proteins in 293FT cells (predicted molecular masses, 36 and
132 44 kDa, respectively), and found that the former migrated as a protein of 73 kDa
133 while the latter migrated at 55 kDa. Sequence analyses showed that the N-terminal
134 half of BEND2 was much more disordered and hydrophobic than the C-terminal half
135 (Fig. S1K). Therefore, it was appropriate that BEND2 displayed slower
136 electrophoretic mobility due to its usual sequence/structure at the N-terminus.

137 By using FLAG-BEND2 KI mice and the mmAb-FLAG antibody, we confirmed
138 that p140 was exclusively expressed in testes among all of the tissues we examined
139 (Fig. 1D). Using immunostaining of FLAG-BEND2 in testicular sections that could
140 be staged based on hematoxylin staining, we found that the protein was highly
141 expressed in preleptotene (plpSCs), leptotene (lepSCs), zygotene (zygSCs), and
142 pachytene (pacSCs) spermatocytes from stages VII to III, and weakly in type B
143 spermatogonia (SG-B) and pacSCs at stages V and VI (Fig. 1E, F). Interestingly,
144 immunofluorescence imaging with higher magnification and a shorter exposure time
145 revealed that the signals for FLAG-BEND2 in the nuclei of spermatocytes were
146 punctate (Fig. 1G). These results indicated that BEND2 is an evolutionarily conserved
147 novel protein, and that it is specifically expressed in the nuclei of spermatogenic cells
148 in a stage-specific manner, shortly before and after meiotic initiation.

149 ***Bend2* gene knockout arrests spermatogenesis at prophase of meiosis I**

150 We endeavored to assess the function of BEND2 by evaluating phenotypic
151 changes in gene knockout (KO) mice that were generated by using CRISPR-Cas9
152 technology (27). Several founder mice with different mutant alleles were acquired,
153 and a female (*Bend2*^{-4k/+}) carrying a mutant allele with a 4-Kbp deletion (-4k)
154 corresponding to a 104-aa deletion in the protein was crossed with WT males to at
155 least the F2 generation for phenotypic evaluation (Fig. 2A–C, S2G). BEND2 was
156 undetectable in the male founder with a 19-bp deletion (*Bend2*^{-19/Y}) or male offspring
157 from the founder with a single-base insertion (*Bend2*^{+1/+}) (Fig. S2A, S2B).

158 The *Bend2* KO male mice were infertile and exhibited markedly smaller testes
159 than their wild-type littermates (Fig. 2D and E and Fig. S2C and D), and fertility
160 testing showed that *Bend2* mutant males were infertile (Fig. 2F and Fig. S2E), and
161 that they did not produce any haploid spermatids or spermatozoa (Fig. 2G, H, S2F). A
162 closer inspection of the H&E-stained testicular sections revealed that the KO testes
163 contained spermatogonia, leptotene and zygotene spermatocytes, and Sertoli cells—
164 but no other type of germ cell (Fig. 2G). We also frequently observed apoptotic cells
165 with condensed nuclei, and their presence was confirmed by TUNEL assays (Fig. 2G,
166 I). Both the numbers of TUNEL-positive tubules and TUNEL-positive cells per tubule
167 were significantly higher than numbers in the wild-type (WT) testes (Fig. 2J). In
168 contrast, the numbers of undifferentiated spermatogonial stem cells (PLZF⁺) and
169 Sertoli cells (WT1⁺) were equivalent between KO and WT mice (Fig. 2K–N). These
170 results indicated that BEND2 plays a specific and essential role in meiosis in male
171 mice.

172 **BEND2 occupies a role in DSB repair and synapsis**

173 We next examined the molecular defects in the KO spermatocytes by immunostaining
174 marker proteins involved in meiosis. The sub-stages of meiotic prophase I that include
175 leptotene, zygotene, pachynema, and diplotene can be distinguished by the co-
176 immunostaining patterns of SYCP3 and the phosphorylated form of histone H2AX
177 (γ H2AX) that marks DSBs formed during meiosis. Under normal conditions, γ H2AX
178 signals in lepSCs and zygSCs are diffusely localized over the nuclei, indicating large
179 numbers of unrepaired DSBs. In contrast, in pacSCs and dipSCs, the signal is a small

180 dot that marks a territory occupied by the X and Y chromosomes (also known as the
181 sex body). The clearance of γ H2AX from the nuclei of pacSCs (except for the sex
182 bodies) indicates that DSBs in the autosomes have been repaired. pacSCs were easily
183 identified in WT testes by the co-staining of γ H2AX and SYCP3, but they were
184 absent in the KO testes (Fig. 3A). As some tubules contained only dot-shaped or
185 diffuse γ H2AX signal while others contain both, three types of tubules (dot-only,
186 diffusion-only, and double-stained) and two types of cells (dot and diffusion) were
187 observed in WT testes (Fig. 3B, C, S3A). In contrast, only diffusion-only tubules and
188 diffusion cells were seen in KO testes. Moreover, the numbers of both diffusion-only
189 tubules and diffusion cells were much higher in KO testes than in WT testes (Fig. 3B,
190 C, S3A). These results indicated that DNA DSB were formed but not properly
191 repaired in *Bend2* KO mice.

192 Co-immunostaining of SYCP3 and γ H2AX was also carried out on surface-
193 spread spermatocytes to reveal details that were invisible in testicular sections (Fig.
194 3D and Fig. S3B). While all spermatocytes from leptotema to diakinesis of meiosis I
195 were observed in WT testes, pacSCs with dot-shaped γ H2AX signals and subsequent
196 cell types were never observed in KO mice (Fig. 3D, Fig. S3B). Moreover, the
197 SYCP3-labeled chromosomal axes in KO zygSCs were not typical of the long
198 continuous threads in WT zygSCs; rather, the former were more condensed, and we
199 therefore named them zygSC-like cells (zygSC-LCs). Quantitative analyses showed
200 that the percentages of lepSCs and zygSC-LCs among all cells at prophase of meiosis
201 I were much higher in KO testes (Fig. 3E). As the diminution in the total number of
202 spermatocytes contributed to the increase in the percentages of lepSC and zygSC-LCs
203 in KO testes, when we calculated the percentages of lepSCs of cells with diffuse
204 γ H2AX signals, we found that the percentage did not change between KO and WT
205 mice (Fig. 3F). This suggested that the absolute number of lepSCs was increased
206 commensurately in KO mice as the total number of cells with diffuse γ H2AX staining
207 per tubule was increased.

208 The progression of synapsis between homologous chromosomes can be
209 monitored by the co-staining of SYCP3 and SYCP1. Under normal conditions,
210 SYCP3 but not SYCP1 can be detected in lepSCs; SYCP1 is initially detectable in
211 zygSCs as short segments along the relatively more continuous SYCP3 threads; and it
212 then becomes fully co-localized with SYCP3 to form the thick, smooth, and
213 individualized synaptonemal complex in pacSCs. Surprisingly, we noted SYCP1 in

214 approximately 29% of KO lepSCs (Fig. 4A, S4A), and we identified three types of
215 zygSC-LCs (zygSC-L1, 46%; zygSC-L2, 28%; zygSC-L3, 26%) in KO testes (Fig.
216 4B). SYCP1 signals were primarily observed as small dots in zygSC-L1, while in
217 zygSC-L2, they were thick or thin segments that represented the synaptonemal
218 complex between homologous chromosomes and sister chromatids, respectively. In
219 zygSC-L3, the SYCP1 signal was mostly detected as 40 discontinuous thin segments
220 representing 40 univalents that underwent synapsis between sister chromatids.

221 As inter-sister synapsis was first uncovered in mice with cohesin gene *Rec8*
222 knockout and that was also observed in KO/mutant mice of several other cohesin
223 genes, we examined whether REC8 foci were modified in *Bend2* KO mice (28).
224 Because the REC8 foci were numerous and not well separated from each other, we
225 measured the distances between well-separated foci along chromosomal axes in
226 zygSCs and zygSC-LCs, and found that the average distance in KO mice was
227 significantly longer than in WT littermates, suggesting a reduced number of REC8
228 foci in zygSC-LCs (Fig. 4D, S4C). Notably, SYCP3 signals in the form of forks,
229 bubbles, and unequal branches were frequently detected in zygSC-L1 (Fig. 4B). The
230 presence of multiple and unequal branches was more evident in images from super-
231 resolution structured illumination microscopy (SIM), and signified synapses between
232 nonhomologous chromosomes (Fig. 4C, S4B). These results suggested that synapses
233 initiated prematurely in KO mice (as early as in lepSCs), but could not be fully
234 established between homologs; instead, they progressed in incorrect directions to form
235 non-homologous and inter-sister synapses in different zygSC-LCs.

236 Meiotic DNA DSBs are repaired in a step-wise manner. The DNA ends of DSBs
237 are resected into long single-stranded 3' overhangs that are initially coated by RPA
238 proteins. RPAs are subsequently replaced by the recombinase proteins RAD51 and
239 DMC1 to form nucleoprotein filaments that seek a homologous template and form the
240 recombinant intermediate, and are finally resolved into either crossovers between
241 homologs or non-crossovers (29, 30). We distinguished the DNA-bound RPA2,
242 RAD51, and DMC1 as hundreds of foci along the SYCP3 threads in lepSCs and
243 zygSCs by co-immunostaining (Fig. S4E–G). In WT mice, the focus numbers of all
244 three proteins increased from leptotema to early zygonema, decreased from early to
245 late zygonema, and reached their nadirs at pachynema (Fig. 4E–G). In lepSCs, both
246 the numbers of foci for RPA2 and DMC1 were similar between WT and KO mice,
247 while the number of RAD51 foci in KO mice was higher than in WT controls. The

248 numbers of RPA2 foci in zygSC-L1 and L2 were similar to those in early and late
249 zygSCs in WT mice, respectively. Of note, the number of RPA2 foci in zygSC-L3 was
250 also similar to that in early zygSCs. The numbers of both RAD51 and DMC1 foci
251 were similar among early zygSCs, zygSC-L1 and zygSC-L2. Of greater interest, the
252 number of RAD51 foci in zygSC-L3s was lower than in late zygSCs but higher than
253 pacSCs while the number of DMC1 foci in zygSC-L3 was the lowest among all cell
254 types. These results suggested the following: 1) that more recombinant intermediates
255 were formed in KO lepSCs than in WT ones, consistent with the appearance of a
256 significant proportion of SYCP1⁺ lepSCs in KO mice; 2) that the formation of
257 recombinant intermediates in zygSC-L1 and zygSC-L2 was relatively normal
258 compared with that in early zygSCs of WT mice, suggesting these two KO cell types
259 are still at the early zygonema with aberrant synapses; 3) that zygSC-L3 underwent
260 inter-sister synapsis with an abnormal and unique DSB repair mechanism as the level
261 of RPA2 foci sustained high while those of RAD51 and DMC1 foci dropped
262 significantly compared with the other two types of zygSC-LCs.

263 **BEND2 interacts with transcriptional suppressors**

264 As BEN proteins were predicted to mediate protein–protein and protein–DNA
265 interactions, and since supportive evidence has been acquired for several family
266 members, we next applied co-IP-mass spectrometry (co-IP-LC-MS/MS) to identify
267 potentially interacting partners of BEND2. Co-IP was conducted using mAb-FLAG
268 to pull down FLAG-BEND2 and its interacting partners from testicular lysates from
269 FLAG-BEND2 KI mice, and testicular lysates from WT mice were used as negative
270 controls. By analyzing proteins enriched in FLAG-BEND2 KI samples in three
271 independent experiments, we identified several potential BEND2-interacting proteins
272 (Fig. 5A).

273 FLAG-BEND2 manifested the highest enrichment rank among all enriched
274 proteins, indicating that our method was reliable. The next top-three most
275 significantly enriched proteins were ZMYM2, ADNP, and KDM1A (also known as
276 LSD1). ZMYM2 is a member of the MYM (myeloproliferative and mental
277 retardation)-type zinc finger protein family that contains six members in the human
278 and mouse genomes (31). LSD1 is the first histone demethylase to be discovered and
279 removes methyl groups from H3K4me or H3K4me2 (32). ZMYM2 has been

280 identified as a component of LSD-containing repressive complexes, including the
281 nucleosome remodeling and histone deacetylase (NuRD) complex (33-35). These
282 complexes typically also contain histone deacetylases such as HDAC1 and HDAC2
283 that act upstream of LSD1 (36). Intriguingly, we found that HDAC1 and 2 were
284 indeed enriched 1.6- and 1.2-fold, respectively, in our FLAG-BEND2 KI samples
285 (Table S1). ADNP is a transcription factor that contains nine zinc fingers and a
286 homeobox domain, and is essential for embryonic and brain development (37, 38). It
287 was reported that ADNP, chromatin remodeler CHD4 (which is the motor component
288 of the NuRD complex), and chromatin architectural proteins HP1 β and HP1 γ formed
289 a stable complex named ChAHP that represses the expression of lineage-specifying
290 genes in ESCs (39). CHD4 consistently ranked No. 7 in the list of BEND2-interacting
291 proteins based upon our results. Other potential interacting proteins that have been
292 uncovered include TAF1B, GTF2H1, and PIWIL2 (40-42).

293 The interactions of BEND2 with ZMYM2, ADNP, LSD1, CHD4, HDAC1, and
294 HP1 γ were confirmed by co-IP-western blotting results using the testicular lysates
295 from FLAG-BEND2 KI mice (Fig. 5B). As positive controls, the interactions between
296 CHD4 and HDAC1, HP1 γ , or ADNP in the testis were also confirmed by our
297 experiments (Fig. 5C, D). We found that CHD4 was abundantly expressed in the
298 nuclei of SG-A and weakly expressed in the nuclei of lepSCs and zygSCs as granules
299 similar to the pattern for BEND2. In *Bend2* KO mice, the signals for CHD4 in lepSCs
300 and zygSC-LCs were dramatically reduced (Fig. 5E). By mining the human PPI data
301 generated using the yeast two-hybrid technique, we also found that human BEND2
302 might interact with ZMYM6, LHX2, SCML2, GTPBP6, and PRR20A-E (43).
303 Therefore, BEND2 appears to interact with a large number of chromatin-binding
304 proteins that are epigenetic regulators and/or transcription factors (Fig. 5F).

305 **BEND2 preferentially binds to simple sequence repeats**

306 To characterize BEND2-binding sites on the genome, we conducted ChIP-seq
307 analyses by using testicular lysates from FLAG-BEND2 KI mice; and based on the
308 data from six independent experiments, a total of 16,477 peaks were identified (Fig.
309 6A, S6A, B; Table S2)—and some peaks were validated by ChIP-PCR. (Fig. S6C). Of
310 note, BEND2 peaks were enriched in proximal promoters (from -1 kb to +100 bp of
311 transcriptional start sites), CpG islands, 5'UTRs, and repetitive sequences ($p < 0.05$)

312 (Fig. 6B, S6D). Almost all peaks (95%) were localized to the intergenic regions (58%)
313 and introns (37%), and these peaks were enriched in simple repeats, low-complexity
314 sequences, and satellites (Fig. 6B, S6D). The order of enrichment-fold values (ratios
315 of observed-to-expected peak numbers) for the enriched genomic regions were simple
316 repeats (15.9), low-complexity sequences (11.0), satellites (1.8), 5'UTRs (1.7), and
317 promoters (1.3). We were interested in whether BEND2 peaks were enriched with any
318 known or novel motifs, and noted that several similar GA-rich motifs were enriched
319 in BEND2 peaks (Fig. 6C, S6E). The top enriched motif
320 (AGGAC/T/AAGGAC/T/AAG) was present in 44% of peaks ($P = 1 \times 10^{-5036}$) (Fig.
321 6C left), and the average intensity (566 reads/peak) of peaks containing the top motif
322 was 2.5-fold higher than peaks without the motif ($P = 1 \times 10^{-267}$). We further
323 conducted motif-enrichment analyses on peaks that contain the top motif and we
324 identified a similar top motif (AA/CG/CGAAAGGAA/TA), and several other known
325 ones (Fig. 6C right, S6E). We observed that this new top motif was similar to the
326 motif for UME1, a protein that associates with histone deacetylases to repress meiotic
327 gene expression during vegetative growth in yeast (44, 45); and to the motif for PU.1,
328 a well-known master regulator and pioneer factor in hematopoiesis from the ETS
329 transcription factor family (46)(Fig. S6E).

330 We additionally generated ChIP-seq data for the BEND2-interacting proteins
331 CHD4, ADNP, and ZMYM2, and found that reads of these BEND2- interacting
332 proteins were enriched at BEND2 peaks (Fig. 6D, E; Table S3). Similar to the case for
333 BEND2, these proteins preferentially bound to proximal promoters, CpG islands,
334 5'UTRs, and repetitive sequences that included simple repeats, low-complexity
335 sequences, and satellites (Table S4). Several differences in genomic-region
336 enrichment were observed for BEND2 and its interacting proteins. First, enrichment-
337 fold values vary. For example, BEND2 peaks were only slightly enriched in proximal
338 promoters 1.3-fold while CHD4, ADNP, and ZMYM2 were enriched in these regions
339 23-, 7-, and 27-fold, respectively. Moreover, BEND2 was enriched in CpG islands
340 two-fold while CHD4, ADNP, and ZMYM2 were enriched 52-, 19-, and 50-fold,
341 respectively. Second, while all of these proteins were only slightly enriched in repeats
342 in general (fold-values no greater than 2), they were enriched in specific repeat-types
343 to greater extents (Fig. 6F, G): fold-values for BEND2, CHD4, ADNP, and ZMYM2
344 in simple repeats were 16, 17, 28, and 8, respectively. ZMYM2 was also enriched in
345 SINEs and LTRs in addition to simple repeats, low-complexity repeats, and satellites.

346 When we examined the genomic distribution of CHD4, ADNP, and ZMYM2 in
347 mESCs using published datasets (Fig. 6F, G) (39, 47, 48), we observed that these
348 proteins were also enriched in promoters, CpG islands, and 5UTRs—except that
349 ADNP was not enriched in 5'UTRs. Moreover, they were enriched in specific repeat
350 types: for example, CHD4 and ADNP were enriched in SINEs and satellites, and
351 ZMYM2 was enriched in SINEs, LTRs, and simple repeats. Notably, almost all
352 BEND2 peaks were enriched in CHD4, ADNP, and ZMYM2 reads, and almost all
353 CHD4 peaks were enriched in ADNP and ZMYM2 peaks, but fewer than half of the
354 CHD4 peaks were enriched in BEND2 reads (Fig. 6D, S6G). Collectively, these data
355 suggested that BEND2 preferentially targets a fraction of its interacting proteins to
356 particular regions (such as simple repeats) in spermatogenic cells.

357 **BEND2 binds to multiple chromatin states**

358 We were interested in discerning how BEND2 sites in the mouse genome were
359 related to different epigenetic markers such as histone modifications. Spruce et al. (7)
360 recently defined an 11-state epigenomic map of leptotene and zygotene spermatocytes
361 by using ChIP-seq data of 10 histone modifications and variants. This was
362 accomplished by using chromHMM, a software package that was based upon a
363 multivariate Hidden Markov Model (49, 50). We reproduced the 11-state map and
364 further annotated the map with more genomic features (RefSeq Genes, CpG islands,
365 and repetitive sequences) and the binding sites of proteins involved in meiosis
366 (PRDM9, REC8, RAD21L, and CTCF) (Fig 6H). Consistent with what Spruce et al.
367 found using a single dataset, we found that PRDM9 sites from three independent
368 datasets were mostly enriched in state 7 (recombination hotspots), which is
369 characterized by the moderate-to-high levels of H3K4me3, H3K36me3, H3K4me1,
370 and H3K9ac. REC8, RAD21L, and CTCF were most enriched in state 1, which
371 represents promoters and insulators, and second most enriched in hotspots. We also
372 mapped BEND2 sites to the state map (Fig. 6H). BEND2 was notably enriched in
373 multiple states, with the highest enrichment in state 8; this is typical of H3K27me3,
374 which is a marker for the Polycomb repressive complex-repressed region. These data
375 suggest that BEND2 thus occupies multiple functions in meiosis, one of which might
376 be transcriptional suppression. ChromHMM results also showed that BEND2 and its
377 interacting partners CHD4, ADNP, and ZYMM2 were enriched in state 1, which is

378 annotated as promoters/insulators. These proteins were additionally enriched in state
379 7, and slightly in states 3–5, which represent enhancers. It is noteworthy that the most
380 enriched states of diverse proteins are usually different, indicating that they do not
381 always remain together. That BEND2 is more or less enriched in many states in
382 spermatocytes suggests that it could be a multifunctional participant in meiosis, and
383 that it likely interacts with different partners in a context/state-dependent manner (Fig.
384 6H, S6H).

385 **BEND2 regulates the expression of a large number of genes**

386 We next executed RNA-seq analyses to identify genes that were regulated by
387 BEND2. We identified 2521 and 3918 genes that were up- and downregulated ($q <$
388 0.05 , $n=4$), respectively, by *Bend2* KO in adult lepSCs and zygSCs (Fig. 7A, S7A and
389 B; Table S5). With an FDR of no more than 0.05, the upregulated genes were enriched
390 in GO terms related to gene regulation (“transcription,” “mRNA transport,” “RNA
391 processing,” “RNA splicing,” “gene silencing,” “chromatin modification,” “regulation
392 of translation,” “methylation”), DNA activities (“DNA replication,” “DNA repair,”
393 “double-strand break repair,” “nucleosome assembly”), and cell cycle (“cell cycle,”
394 “cell division,” “mitotic nuclear division,” “cell proliferation”) (Fig. 7B, Table S5).
395 Such enrichments are typical of genes that are highly expressed in spermatogonia (26,
396 51, 52), and that are described as “somatic/progenitor program” genes by Hasegawa et
397 al. as they are commonly active in somatic lineages and mitotic phases of
398 spermatogenesis-progenitor cells (19). Some of these upregulated genes (*Ehmt2*,
399 *Nanos3*, *Mtor*, *Tdrd1*, *Dazl*, *Lin28a*, *Wdr81*, *Msh2*, *Erc1*, *Kit*, *Asz1*, *Dnd1*, *Mov10l*,
400 *Bax*, *Piwil2*, *Stra8*, *Kmt2d*, *H3f3b*, *Sohlh1*, *Src*, *Sohlh2*, *Adrm1*, and *Trip13*) are
401 annotated as “germ cell development” or “oogenesis” (Table S5). GO terms enriched
402 in the downregulated genes were quite different, and they were mostly related to
403 meiotic or post-meiotic activities such as “spermatogenesis,” “sperm motility,” “cell
404 differentiation,” “cilium,” and “capacitation.” By replotting the expression of these
405 genes using our previous RNAs-seq data, we found that the upregulated genes were
406 indeed expressed at higher levels in spermatogonia than in spermatocytes, while the

407 downregulated genes exhibited the opposite expression pattern (Fig. S7D). Therefore,
408 it appears that one of the BEND2 functions is to terminate the somatic/progenitor
409 program and to promote the expression of meiotic and post-meiotic genes.

410 We next investigated whether BEND2 regulated gene expression by affecting
411 chromatin accessibility and/or modifications. We isolated zygSCs to implement
412 ATAC-seq and ChIP-seq analyses for H3K4me3 and H3K27me3, and to compare
413 differences in the distributions and intensities of the three signals between WT and
414 *Bend2* KO samples. From the chromHMM state map, we observed that all three types
415 of peaks in both WT and KO mice were mostly enriched in promoters followed by
416 hotspots (states 1 and 7) (Fig. 6H). We did not note any changes in their global
417 enrichment patterns by *Bend2* gene KO from the state map. However, by examining
418 the signal intensities around transcription start sites (TSSs), we observed that ATAC-
419 seq signals were enhanced in a large number of genes (clusters 1 and 2 versus cluster
420 3) in KO mice (Fig. 7C, Fig. S7H), and the H3K4me3 but not H3K27me3 signals
421 were also enhanced in clusters 1 and 2 (Fig. 7D, Fig. S7I). When we assessed whether
422 up- or downregulated genes based on RNA-seq were enriched in any of these three
423 clusters, we found that only the upregulated genes were enriched in clusters 1 and 2 (P
424 = 2.0×10^{-32} and 1.0×10^{-38} , respectively) (Fig. 7E). Therefore, it appeared that gene
425 repression by BEND2 was achieved by its contribution to maintaining low levels of
426 chromatin accessibility and H3K4me3.

427 Finally, we carried out luciferase assays to examine whether BEND2 suppressed
428 gene expression by binding to genomic regions identified by the ChIP-seq analyses
429 (Fig 7F-G). We first synthesized a DNA fragment containing five copies of the
430 GGAAA consensus motif and inserted it upstream of the basal promoter of the
431 luciferase-expressing plasmid. Intriguingly, the 5xGGAAA sequence enhanced the
432 promoter activity by itself, and BEND2 enabled this enhancement to revert to basal
433 levels. Based on the ChIP-seq data, we next tested several putative native BEND2-
434 binding sites that were located either around the TSS (*Suz12*, *Lin28a*, *Dmrt1*) or in the
435 gene body (*Exo1*) of genes that were upregulated in *Bend2* KO mice, and we observed
436 that the overlapping or nearby ATAC-seq and H3K4me3 peaks were also upregulated

437 in KO mice. BEND2 significantly reduced the activities of three of the four putative
438 binding sites (*Suz12*, *Lin28a*, *Exo1*), regardless of whether the sites by themselves
439 augmented or attenuated the activity of the basal promoter in the luciferase plasmid
440 (Fig. 7G). These results further supported the concept that BEND2 functions as a
441 transcriptional suppressor of certain genes by binding to and modifying the chromatin
442 accessibility and histone modifications of particular genomic regions.

443 **DISCUSSION**

444 Meiosis is a highly complex process that entails numerous concurrent or
445 sequential steps that must be coordinated by a large number of regulators. And
446 investigators have in recent years repeatedly described novel regulators of meiosis
447 using phenotypic evaluation of their gene KO mice via the highly efficient
448 CRISPR/Cas9-based gene-editing technology. In the present study, we identified
449 BEND2 as another novel regulator that is specifically expressed in male germ cells
450 shortly before and after meiosis initiation, and that is essential for DSB repair and
451 synapsis using gene KI and KO mice. We also demonstrated that BEND2 interacts
452 with other chromatin-binding/regulating proteins and regulates chromatin state and
453 transcription. Our work has thus contributed another significant component to the
454 arcane and complex physiologic process that is meiosis.

455 **The BEN protein family**

456 The BEN protein family is a relatively new family that was discovered using
457 bioinformatic analyses, and studies on this family are limited. BANP (BTG3
458 associated nuclear protein, also known as scaffold/matrix-associated region 1
459 (SMAR1, or BEND1), has been reported to act as both a tumor suppressor and
460 immunomodulator (53), and to repress cyclin D1 expression by recruitment of the
461 SIN3/HDAC1 complex to its promoter and to direct histone modifications from a
462 distance (54). E5R is a virosomal protein from the chordopoxvirus subfamily and
463 likely plays a role in organizing viral DNA during replication or transcription. NAC1
464 (nucleus accumbens-associated protein 1, also called NACC1 or BEND8) participates
465 in various biological processes that include neuronal activity, pluripotency of ESCs,

466 and tumor growth, and it interacts with HDAC3, HDAC4, and REST corepressor 1
467 (CoREST) (55, 56). NAC1 was recently reported to bind DNA directly through the
468 BEN domain in a sequence-specific manner (57).

469 BEND3 contains four BEN domains; is associated with HP1 α , HP1 β , HP1 γ , and
470 H3K9me3-containing heterochromatic foci; and represses transcription through
471 interactions with HDAC1, 2, 3, and SALL4—a transcriptional repressor that also
472 associates with the NuRD complex (58). In the absence of DNA methylation or
473 H3K9me3 in mouse ESCs, BEND3 recruits the MBD3/NuRD complex to
474 pericentromeric regions and is necessary for PRC2 recruitment and H3K27me3
475 establishment at major satellites, suggesting that it is a key factor in mediating a
476 switch from constitutive to facultative heterochromatin (59). BEND3 also represses
477 rDNA transcription by interacting with the nucleolar remodeling complex (NoRC)
478 (60) and with Suv4-20h2, an enzyme responsible for H4K20 trimethylation (61).
479 BEND5 and BEND6 contain a single BEN domain and are therefore (together with
480 three fly proteins) called “BEN-solo” factors (62). BEND6, similar to its *Drosophila*
481 homolog Insensitive, is most abundantly expressed in the brain and inhibits Notch
482 target genes (63, 64). Importantly, BEND5 and the fly BEN-solo factors bind directly
483 to specific DNA motifs through their BEN domains (62, 65). BEND9 (NACC2, RBB)
484 recruits the NuRD complex to the internal promoter of HDM2 and inhibits the
485 expression of HDM2, an E3 ligase that specifically targets p53 for ubiquitination and
486 subsequent degradation (66). These studies on the BEN family members have
487 revealed the following: 1) the BEN proteins tend to interact with a variety of proteins,
488 presumably in a context-dependent manner; 2) most of the interacting partners are
489 components of transcription-repressive complexes involved in chromatin remodeling
490 and/or modification; and 3) the BEN proteins can also act as sequence-specific DNA-
491 binding proteins.

492 We have uncovered little information regarding BEND2, 4, and 7. Several
493 studies showed that in-frame MN1-BEND2, EWSR1-BEND2, and CHD7-BEND2
494 gene fusions were detectable in brain and pancreatic neuroendocrine tumors (67-69).
495 The MN1 (meningioma 1) gene is a proto-oncogene that encodes a transcriptional
496 regulator, and its mutations and abnormal expression are frequently detected in tumors
497 (70). EWSR1 (EWS RNA-binding protein 1) is a multifunctional protein that
498 regulates transcription and RNA splicing, and occupies diverse roles in various

499 cellular processes and organ development— including meiosis (71). And CHD7
500 (chromodomain helicase DNA-binding protein 7) is a chromatin-remodeling enzyme
501 involved in differentiation and transcriptional regulation (72, 73). The current
502 observations that all fusion partners of BEND2 are transcriptional regulators and that
503 the fusion proteins maintain the BEN domain of BEND2 suggest that BEND2
504 probably binds DNA through its BEN domain.

505 **BEND2 protein and its expression**

506 In the present study, we found that BEND2 was specifically expressed in male germ
507 cells after birth. This conclusion was supported by immunostaining results where we
508 used a rabbit polyclonal antibody against a BEND2-specific polypeptide outside of
509 the two BEN domains, and a mouse monoclonal antibody against the 3xFLAG tag
510 fused to the N-end of BEND2 of the mice with FLAG-BEND2 KI. One interesting
511 observation was that the molecular mass of BEND2 as determined by SDS-PAGE was
512 much greater than expected from the amino acid number. This discrepancy may have
513 resulted from the highly disorganized structure and high hydrophobicity of the
514 predicted protein sequence from bioinformatic analyses. However, we cannot rule out
515 the possibility that the protein was post-translationally modified. The punctate signals
516 of BEND2 in lepSCs (Fig. 1G) that did not co-localize with brighter DAPI signals
517 suggested that BEND2 molecules tend to aggregate in regions that are not typical
518 constitutive heterochromatin. We also failed to detect BEND2 in spermatocyte
519 chromosomal spreads and to express the full-length protein in bacteria, suggesting
520 that the BEND2 protein molecules and their putative associations with each other and
521 with other molecules are likely to be labile.

522 **The phenotypes of *Bend2* KO mice**

523 As expected from the male germ-cell-specific expression of *Bend2*, the KO mice
524 displayed male infertility without any other overt phenotypes; this conclusion was
525 solidly supported by the observation that all three types of mutant male mice (*Bend2*^{-19/Y},
526 *Bend2*^{+1/Y}, *Bend2*^{-4k/Y}) were infertile. Detailed phenotypic evaluation was carried
527 out with the *Bend2*^{-4k/Y} males at and after F2 (primarily at F5 when the genetic
528 background was purified to 98% C57/BL6 by repeated crosses between *Bend2*^{+/-}

529 females and C57BL/6J males). Similar to the phenotypes manifested by mice with
530 KOs of many other key meiotic regulators, *Bend2* KO mice exhibited arrested
531 spermatogenesis at the zygonema/pachynema transition, with aberrant DSB repair and
532 chromosomal synapsis. The manifestations of several defects are noteworthy, as they
533 imply molecular functions of BEND2 that warrant further investigation. First,
534 synapsis initiation as marked by the appearance of SYCP1 signals was detectable in a
535 significant portion (~30%) of the KO lepSCs, while it was rarely observed in WT
536 cells. However, the formation of the invasive single-stranded DNAs essential for
537 recombination and synapsis were basically not affected as indicated by the numbers of
538 RPA, RAD51, and DMC1 foci. Second, non-homologous synapses were frequently
539 seen in KO zygSCs. Non-homologous chromosomal association has been reported in
540 mice with KOs of a number of epigenetic regulators such as SUV39H, DNMT3L, and
541 PRDM9, and was likely caused by the disrupted heterochromatin structure (14, 15,
542 74). Third, inter-sister synapses were common in our *Bend2* KO zygSCs, and inter-
543 sister synapses were observed in cohesion-protein KO mice, including KOs of REC8,
544 SMC1 β , and STAG3(75). REC8 foci were also consistently reduced in *Bend2* KO
545 mice. The phenotypes of *Bend2* KO mice might therefore reflect those of different
546 meiotic regulators, and this implies that BEND2 is a multifunctional protein that is
547 involved in several processes/steps of meiosis.

548 **The molecular functions of BEND2**

549 The first clue as to the molecular mechanism underlying BEND2 function in
550 meiosis comes from the observation that it interacts with multiple proteins, of which
551 most are transcriptional repressors that often interact with other BEND proteins. The
552 majority of these proteins (HDAC1, HDAC2, CHD4, LSD1, ZMYM2, HP1g, and
553 ADNP) are either core components or interacting proteins of two repressive
554 complexes: the well-known NuRD complex (76) and the recently identified ChAHP
555 complex (39). This observation together with the fact that our co-IP washing solution
556 contained 500 mM NaCl suggested that the interactions between BEND2 and these
557 complexes were fairly robust. The overlapping expression windows of BEND2 and
558 CHD4, their similar granular expression patterns, and the reduced signal for CHD4 in
559 *Bend2* KO mice also supported an interaction between these two proteins. The
560 interaction between BEND2 and CHD4 and the fact that CHD4 is involved in

561 regulating DSB repair (77, 78) facilitate the clarification of why and how meiotic
562 DSB repair was disrupted in *Bend2* KO mice. Other familiar interacting proteins of
563 BEND2 that we identified in the present study included DNA-binding proteins such
564 as TAF1B, GTF2H1, and PIWIL2. Based on our data and bioinformatic predictions
565 from the PPI database, BEND2 interacts with many other proteins; however, the
566 significance of these interactions is currently unclear. Nevertheless, these observations
567 suggested that BEND2, like other family members, may be important in regulating
568 chromatin activities such as heterochromatin formation/maintenance, transcription
569 and higher-order structure.

570 The potential regulatory roles of BEND2 with respect to chromatin are also
571 supported by the distribution of its ChIP-seq peaks in various genomic regions and the
572 chromatin states as defined epigenetically. The peaks were enriched in regulatory
573 regions such as promoters, CpG islands, enhancers, recombination hotspots, and PRC-
574 repressed sites. It was particularly interesting that the intronic and intergenic peaks
575 (which comprised 96% of the total peaks) were highly enriched in simple repeats and
576 low-complexity repeats, and that a GA-rich motif was also enriched in these peaks.
577 Compared with other chromatin regulators such as CHD4, ADNP, and ZMYM2 that
578 were mainly enriched in promoters and hotspots (Fig. 6H), BEND2 was enriched in
579 almost all of the regulatory regions and heterochromatin types. BEND2 was only
580 slightly enriched in proximal promoters (1.3-fold), while the other factors were highly
581 enriched (23-, 7-, and 27-fold for CHD4, ADNP, and ZMYM2, respectively). In
582 contrast, enrichment of these proteins in simple repeats was comparable (16, 17, 28,
583 and 8, respectively). However, the types of simple repeats in which CHD4, ADNP,
584 and ZMYM2 were enriched in spermatocytes were somewhat different from those in
585 mESCs. These observations suggested that BEND2, as a germ cell-specific
586 chromatin-binding protein, either guides or stabilizes the genomic distribution of its
587 interacting partners. More evidence for this hypothesis will only be acquired from
588 future studies by examining the distributional changes in these partners in *Bend2* KO
589 cells or in mESCs in which exogenous BEND2 was expressed.

590 **Regulation of gene expression by BEND2**

591 The analyses of transcriptomic changes in spermatocytes of *Bend2* KO mice

592 provided further clues regarding the molecular functions of BEND2 as a chromatin
593 regulator. It appears that BEND2—similar to Sex comb on midleg-like 2 (SCML2)—
594 also contributes to shutting down the mitotic program and to activating or enhancing
595 the meiotic and post-meiotic program of spermatogenic cells (19). As SCML2 was
596 expressed in cells ranging from undifferentiated spermatogonia to spermatocytes, and
597 as global gene-expression changes were not observed until the spermatocyte stage
598 upon *Scml2* KO, these authors proposed that the suppressive process was initiated
599 prior to when the effect became obvious after meiotic initiation. This indicated that
600 some components of the SCML2-involved suppressive machinery were not ready
601 until the late stage of spermatogonial differentiation, and therefore BEND2 may be
602 one of the missing components as its expression was noted just prior to meiotic
603 initiation. Therefore, it would be compelling to investigate the relationship between
604 SCML2- and BEND2-mediated suppressive machineries in the future.

605 Increasing evidence signifies that gene repression is an essential means of gene
606 regulation in diverse cellular developmental processes. As far as germ cells are
607 concerned, somatic genes are repressed in early-stage primordial germ cells in both
608 sexes (79), while the meiotic program is prevented in male gonocytes and
609 spermatogonia by the RA-metabolizing pathway (80) and proteins such as NANOS2,
610 DMRT1, and SCML2 (19, 81, 82). Thus, the identification of BEND2 as a novel
611 repressive regulator indicates that this regulatory scheme may be much more
612 extensive and complex than previously thought. Since a repressor can specifically
613 activate the expression of genes as an indirect result of the repression of other
614 repressors that target the activated genes, it is not surprising that a large group of
615 genes involved in meiotic and post-meiotic activities of spermatogenic cells can be
616 downregulated upon *Bend2* KO. This suggests that BEND2 contributes to the
617 expression of these genes under normal conditions, and among the genes normally
618 repressed by BEND2 (upregulated genes in *Bend2* KO mice), 109 were negative
619 regulators of transcription according to their GO annotations (FRD=0.04; Table S5,
620 line 37). Repressive regulators that are familiar to us in this list include
621 EHMT1/GLP1, EHMT2/G9A, SALL1, SALL4, DNMT1, DNMT3B, SUV39H2,
622 HDAC2, BEND3, and DMRT1; and some of these are known to be of critical
623 significance to spermatogonial proliferation, differentiation, and meiotic initiation.
624 For example, DMRT1 is a repressor of meiotic initiation as its gene KO in mice

625 initiates meiosis precociously (82). PRC2, of which SUZ12 is a core subunit, is
626 required for spermatogonial stem cell maintenance and meiotic progression via
627 repression of somatic and meiotic gene expression (83). Moreover, we consistently
628 showed with luciferase assays that the TSSs of both *Dmrt1* and *Suz12* contained
629 BEND2-binding sites that were repressive in the presence of BEND2.

630 In summary, we identified BEND2 as a germ-cell-specific regulator of meiosis
631 with detailed examinations of its expression and function by using gene KI and KO
632 mice. We also demonstrated the molecular mechanisms underlying BEND2's action
633 as a chromatin modulator and transcriptional repressor by identifying and
634 characterizing its interacting partners, genomic binding sites, and regulated genes.
635 However, there are many more issues that await clarification in the future. For
636 example, there are the questions of whether BEND2 is critical to female meiosis
637 (oogenesis), whether it acts as a direct modulator of the meiotic machinery
638 independent of its role in transcriptional repression, and whether molecular defects
639 can be detected earlier, prior to meiotic initiation. We posit that the results of our
640 comprehensive present study will establish a solid foundation for such future
641 investigations.

642 **Materials and Methods**

643 **Animal care**

644 All animal procedures were approved by the Animal Ethics Committee of the Institute
645 of Zoology, Chinese Academy of Science. The mice were housed in a specific
646 pathogen-free facility with a 12 h:12 h light-dark artificial-lighting cycle, with lights
647 off at 19:00, and were housed in cages at a temperature of 22–24°C. All experiments
648 with mice were conducted in accordance with the Guide for the Care and Use of
649 Laboratory Animal Guidelines.

650 **RT-PCR**

651 Approximately 50 milligrams of mouse tissue was incubated with 1 ml of TRIzol
652 reagent (Invitrogen Cat. no. 15596-026) and homogenized with a Dounce
653 homogenizer. All liquid was transferred to RNase-free Eppendorf tubes and incubated

654 at room temperature for 5 min. We then added 0.2 ml of chloroform, capped the tubes
655 securely, shook them by hand for 15 s, incubated the tubes for ~3 min at room
656 temperature, and centrifuged them at 12,000 x g for 10 min at 4°C. Supernatants were
657 transferred to a new tube and RNA was extracted using chloroform followed by
658 isopropanol precipitation. The RNA was dissolved in nuclease-free water (P1195,
659 Promega), and the RNA quality was measured with a Nanodrop 2000. To prepare the
660 cDNA library, total RNAs were reverse-transcribed using a High-Capacity cDNA
661 Reverse Transcription Kit (4368814, Applied Biosystems). The primers we used to
662 detect gene expression levels are listed in Table S7. The following conditions were
663 used for PCR: 94°C, 2 min; 30 cycles of 94°C, 1 min; 60°C, 1 min; 72°C, 40 s; and a
664 final extension at 72°C, 10 min. PCR products were separated on 1.5% agarose gels.

665 ***Bend2* cDNA clone**

666 Total RNA was extracted from adult mouse testis and reverse-transcription was
667 performed as described above. We used nested PCR for the *Bend2* cDNA clone (two
668 pairs of primers were designed and their sequences are provided in Table S7). The
669 PCR products were purified with agarose gel electrophoresis and recovered with an
670 EasyPure quick gel extraction Kit (M2073, TransGen Biotech). The cDNA was cloned
671 into a pGM-T plasmid (VT202-02, TIANGEN Biotech), and the cDNA sequence was
672 identified by Sanger sequencing.

673 We identified two transcripts when we attempted to clone the cDNA from mouse
674 testes: one (V1) contained the 14 predicted coding exons, while the other (V2) was
675 missing the 4th exon that corresponded to a 35-aa (105 bp) in-frame deletion in the
676 protein (Fig. S1C). Based on the intensities of the cDNA bands in the stained agarose
677 gel, V1 was more abundant than V2.

678 **Generation and genotyping of *Bend2* mutant mice**

679 *Bend2* mutant mice were generated through the CRISPR/Cas9 gene-editing approach
680 (27). One male and three female founder mice with DBA/2/C57BL/6J background
681 were acquired by using different gRNAs. For *Bend2*^{-4k/Y} mice, we designed two
682 gRNAs for the long-fragment deletion using methods described previously (84), while
683 only one gRNA was designed for the generation of *Bend2*^{+1/Y} and *Bend2*^{-19/Y} mice.

684 Genomic DNA extraction followed the standard proteinase-K-chloroform method.
685 Genotyping for *Bend2*^{+1/Y} and *Bend2*^{-19/Y} mice was executed by Sanger sequencing
686 following PCR amplification, while two pairs of primers were designed for genomic
687 identification of *Bend2*^{-4k/Y} mice; p1 and p2 primers were used to identify the WT and
688 KO allele, respectively (all primers are listed in Table S7).

689 **Generation of anti-BEND2 antibody**

690 Antibodies to mouse BEND2 were produced by ABclonal Technology (Wuhan,
691 China), and generated by immunization of rabbits with the following peptides: 1-30aa
692 (MESDTDDSHISYDGDELFSDFGSDIEDTS-C), and 585-614aa
693 (DVRESVKRERVDFEHTPDANPEGSDNASIN-C). Antibodies were purified using
694 antigen-specific affinity columns.

695 **Generation and genotyping of *Bend2*-3xFLAG knock-in mice**

696 *Bend2*-3xFLAG knock-in mice were generated through the CRISPR/Cas9 gene-
697 editing approach (85). The targeting fragment was designed to insert 3xFLAG in-
698 frame with the coding sequence just after the first ATG of the *Bend2* genomic locus.
699 To ensure accuracy, we designed two gRNAs and compared their efficiencies: KI-
700 gRNA-2 was more efficient and is listed in Table S7. The donor DNA contained a
701 3xFLAG-Linker, with left and right homology arms (800 bp). Donor DNA was
702 synthesized by Sangon Biotech. Genomic DNA extraction also following the standard
703 proteinase K-phenol/chloroform method. PCR was then performed to identify
704 genotype (primers are listed in Table S7).

705 **Histology, hematoxylin and eosin (H&E) staining, immunohistochemistry,** 706 **immunofluorescence, and TUNEL staining in testicular sections**

707 Testes or epididymides from WT and KO mice were dissected and fixed with Bouin's
708 solution or 4% paraformaldehyde (PFA), and then embedded in paraffin and sectioned
709 at 5 µm for staining. For H&E staining, Bouin's solution- fixed sections were stained
710 with H&E following standard protocol. For immunofluorescence or
711 immunohistochemical study, 4% PFA-fixed sections were dewaxed and rehydrated,
712 and then slides were incubated with sodium citrate buffer (pH 6.0) at 95°C for 10 min

713 to retrieve antigen. Five percent BSA or 5% skimmed milk was used to block
714 nonspecific antigens for 1 h at room temperature (RT). Primary antibodies were
715 diluted with 5% BSA and then incubated with sections at 4°C overnight. After
716 washing three times with PBS, diluted secondary antibodies conjugated with
717 fluorescent tag or HRP were incubated with sections. For immunofluorescence, DNA
718 was stained with DAPI diluted by PBS, and photomicrographs were taken with
719 confocal fluorescence microscopes (LSM780, Zeiss; LSM880, Zeiss; Nikon A1 N-
720 SIM S, Nikon). For immunohistochemistry, a DAB solution as chromogen was
721 diluted and used to cover the sections at RT for 10 min; this was immediately
722 followed by PBS to stop the reaction. Slides were dehydrated and nuclei were stained
723 with hematoxylin. Images were taken with an optical microscope (ECLIPSE 80i,
724 Nikon). We implemented the DeadEnd™ Fluorometric TUNEL System (G3250,
725 Promega) for TUNEL staining.

726 **Preparation of tissue extracts and western immunoblotting analysis**

727 Tissues were harvested and washed once with PBS. After mechanically shearing them
728 into pieces, we transferred the tissues to a Dounce for homogenization in RIPA lysis
729 buffer (P0013B, Beyotime) containing protease-inhibitor cocktail, and the mixture
730 was incubated on ice for 30 min. Cell debris was removed by centrifugation at 13,500
731 x g for 15 min at 4°C, and lysates were boiled with 5x SDS loading buffer for 10 min.
732 Tissue extracts were electrophoresed on SDS-PAGE gels containing different
733 concentrations of separation gels between 6% and 15% based upon the molecular
734 weights of the proteins, and then blotted onto PVDF membranes (88518, Thermo).
735 Membranes were blocked with 5% skimmed milk for 1 h at room temperature, and
736 then incubated in dilutions of primary antibodies overnight at 4°C. After washing
737 three times with PBST, membranes were incubated in horseradish peroxidase (HRP)-
738 conjugated secondary antibodies (diluted in PBS) for 1 h at room temperature, and the
739 membranes were then washed three times with PBST at room temperature with gentle
740 shaking. The protein blots were ultimately detected with SuperSignal™ West Pico
741 Plus Chemiluminescent Substrate (34577, Thermo) and imaged on a Bio-Rad
742 Universal Hood II imaging system.

743 **Spermatocyte chromosome spreads and immunofluorescence of spermatocytes**

744 Spermatocyte chromosome spreads of the testicular samples were performed using the
745 drying-down technique (86). Briefly, the testes were dissected from 2–4-month-old
746 mice, and the seminiferous tubules were washed in PBS. The tubules were then placed
747 in a hypotonic extraction buffer for 30–60 min. Subsequently, the tubules were
748 minced in 0.1 M sucrose (pH 8.2) on a clean glass slide and pipetted repeatedly to
749 create a cellular suspension; the suspensions were then spread on slides containing 1%
750 PFA and 0.15% Triton X-100 (pH 9.2), and dried for at least two hours in a closed box
751 with high humidity. Finally, the slides were washed twice with 0.4% Photo-Flo 200
752 (Kodak), dried at room temperature, and stored at -80°C for immunofluorescent
753 staining. Slides were equilibrated to RT, and then each was washed with PBS twice
754 for 5 min with gentle shaking. BSA (5%) was dropped onto the slides for blocking,
755 and they were covered by parafilm for one hour in a humidified box. Fluorescence
756 staining was identical to that described for immunofluorescence staining.
757 Immunolabeled nuclei with chromosomal spreads were imaged on confocal laser
758 scanning microscopes (LSM780, Zeiss; LSM880, Zeiss) using a $63\times$ oil-immersion
759 objective. For SIM, images were taken on a Nikon A1 N-SIM S microscope.

760 **Co-IP-mass spectrometric analyses**

761 Four testes from 15-dpp *Bend2-3xFLAG* knock-in mice or WT mice were
762 homogenized by using Dounce homogenizers in 1 ml of cold lysis buffer (20 mM
763 Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 5% glycerol, and 1% NP-40, with
764 fresh 100x proteinase inhibitor added just before use), and then incubated on ice for
765 30 min. We removed cellular debris by centrifugation at 13,500 g for 15 min at 4°C ,
766 and cell lysates were precleared with 25 μl of protein G beads (10003D, Invitrogen) at
767 4°C for one hour. For mass spectrometry, 80 μl of Anti-FLAG Magarose Beads
768 (SM00905, SMART LIFESCIENCES) were added to the precleared lysates and the
769 mixture rotated at 4°C overnight. The beads were washed four times with cold, low-
770 salt co-IP wash buffer (20 mM Tris-HCl [pH 7.4], 300 mM NaCl, 1 mM EDTA, 5%
771 glycerol, and 1% NP-40, with fresh 100x proteinase inhibitor added just before use) or

772 high-salt co-IP wash buffer (20 mM Tris-HCl [pH 7.4], 500 mM NaCl, 1 mM EDTA,
773 5% glycerol, and 1% NP-40, with fresh 100x proteinase inhibitor added just before
774 use), rotating each time for 15 min at 4°C. Proteins were eluted from the beads with
775 25 µl of 1x SDS loading buffer and boiled for 10 min. The presence of proteins in the
776 immunoprecipitated samples was confirmed by SDS-PAGE using a 10%
777 concentration of separation gel and silver-staining. Whole samples collected from the
778 gel were used to perform mass spectrometric analyses. For co-IP western blotting, 80
779 µl of precleared lysates mixed with 5x SDS loading buffer were boiled for 10 min as
780 an input sample prior to immunoprecipitation. The other lysates were incubated with 5
781 µg of antibodies or isotype IgG as experimental samples and negative control,
782 respectively, and rotated at 4°C overnight. We then added 30 µl of Dynabeads Protein
783 A/G (10001D, 10003D, Invitrogen) to each sample based on the host species of
784 antibodies, and incubated the samples at 4°C for four hours. The washing and elution
785 steps were the same as described above.

786 **In-gel digestion of proteins**

787 The protein bands in each lane were cut into small plugs, washed twice with 200 µl of
788 distilled water, dehydrated with acetonitrile for 10 min, and dried in a Speedvac for
789 approximately 15 min. Gel plugs were treated with 10 mM DTT in 25 mM NH₄HCO₃
790 for 45 min at 56°C for subsequent reactions, and alkylated with 40 mM iodoacetamide
791 in 25 mM NH₄HCO₃ for 45 min at room temperature in the dark, followed by two
792 washes with 50% acetonitrile in 25 mM NH₄HCO₃. The gel plugs were ultimately
793 dried and digested with trypsin (40 ng for each band) in 25 mM NH₄HCO₃ overnight
794 at 37°C. We added formic acid to the reaction buffer for a final concentration of 1% in
795 order to stop the enzymatic reaction. The solution was then transferred to a sample
796 vial for LC-MS/MS analysis.

797 **Rank product analysis and false-positive rate**

798 We determined significance levels for each protein after MS using rank product (RP)

799 analysis (87) and false-positive rate (FDR). First, we merged three replicates of
800 MS_ratio values (from our MS results) by replacing missing values with 1 and kept
801 those genes that reflected an MS_ratio in all thrice-repeated MS experiments. We then
802 ranked MS_ratio values from large to small and calculated rank products for each
803 gene using the following formula: Rank Product = (rank1/n) * (rank2/n) * (rank3/n),
804 where n was the total number of genes, rank1 the rank in the first MS experiment,
805 rank2 the rank in the second MS experiment, and rank3 the rank in the third MS
806 experiment. As a result, each gene had a rank product value based on thrice-repeated
807 MS experiments, and as with a significant p-value, the smaller the rank product score,
808 the more significant the gene. Finally, we computed a false-positive rate (FDR) for
809 each rank product with the Benjamini–Hochberg procedure (88) using the p.adjust()
810 function in the R program. The input value was the rank product value for each gene,
811 and by setting the parameter “method” as “fdr” and “n” as the total number of genes
812 that we considered, we achieved a false-positive rate for each rank-product value.

813 **Sample preparation for RNA-seq**

814 We collected leptotene or zygotene spermatocytes via FACS (89). Briefly, the
815 testes from one adult WT mouse or from three adult KO mice were digested by two-
816 step methods. Seminiferous tubules were segregated with collagenase I and DNase I,
817 and then 0.25% trypsin and DNase I were used to obtain a single-cell suspension.
818 Testicular cells isolated from WT and KO mice were then sorted by FACS after
819 Hoechst 33342 staining. Different types of spermatocytes were then collected through
820 Hoechst Blue and Hoechst Red channels, and RNA was prepared following the
821 TRIzol (Invitrogen) protocol. All RNA libraries were constructed at the same time
822 using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (E7760)
823 according to the manufacturer’s recommendations, and oligo (dT) beads (NEB) were
824 used to isolate poly (A) mRNAs.

825 **ChIP-seq**

826 Approximately 60 mg of testicular tissues from mice at 15 or 40 dpp was

827 disaggregated by Dounce homogenization and the chromatin was crosslinked in PBS
828 containing 1% formaldehyde for 10 min at RT. Fixation of chromatin was halted with
829 a 1.25 M glycine solution and washed three times with cold PBS. Cells was lysed for
830 30 min on ice by adding cell-lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA,
831 and 1% SDS, and fresh 1 mM PMSF and protease inhibitor were added before use).
832 Chromatin was then sonicated for 20 s at 25% power in 30-s pulses for 20 cycles,
833 cellular debris was removed by centrifugation, and the supernatant was precleared by
834 IgG for 2 h at 4°C. About 1/20th of the chromatin was saved as an input sample, and
835 the remainder was diluted to 10x volume with IP dilution buffer (20 mM Tris-HCl
836 [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.01% SDS), and
837 incubated with 10 µg of antibody for 2 h at 4°C. Protein A/G (10001D, 10003D,
838 Invitrogen) Dynabeads were added to capture targeted chromatin overnight. Beads
839 were washed with a four-step wash buffer (low-salt wash buffer, 20 mM Tris-HCl
840 [pH 8.0], 2 mM EDTA, 50 mM NaCl, 1% Triton X-100, and 0.1% SDS; high-salt
841 wash buffer, 20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 500 mM NaCl, 1% Triton X-
842 100, and 0.01% SDS; LiCl wash buffer, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA,
843 0.25 M LiCl, 1% NP-40, and 1% deoxycholic acid; TET buffer, 10 mM Tris-HCl [pH
844 8.0], 1 mM EDTA, and 0.1% Tween 20); beads were washed twice for each step.
845 After the chromatin was eluted from the beads by elution buffer (10 mM Tris-HCl
846 [pH 8.0], 1 mM EDTA, and 1% Tween 20), cross-linking was reversed with 5 M
847 NaCl at 65°C for 16 h; and RNA and protein were digested by adding RNase A and
848 protease K for 2 h at 37°C and 45°C, respectively. Extracted DNA was used to
849 construct a library with the NEBNext Ultra II DNA library Prep Kit for Illumina
850 (E7645, NEB), and qualified libraries were sequenced with an Illumina Novaseq 6000
851 to obtain paired-end 150-nt reads.

852 **CUT&RUN**

853 We collected zygotene spermatocytes via FACS as described above, and CUT&RUN
854 was primarily performed according to Henikoff et al. (90). In brief, before

855 experimentation, 10 μ l of concanavalin A beads (BP531, Bangs Laboratories) were
856 washed twice with binding buffer, resuspended with 10 μ l of binding buffer, and
857 maintained on ice. For each sample, ~20,000 cells were suspended in wash buffer,
858 incubated with prepared beads, and mixed for 10 min at RT. After discarding liquids,
859 beads were incubated with antibody buffer on a Thermomixer at 4°C overnight. Beads
860 were washed once with Dig-Wash buffer, resuspended with the same buffer
861 containing pAG-MNase at a final concentration of 700 ng/ml (we purified the pAG-
862 MNase according to methods described previously (91), and this mixture was
863 incubated for three hours at 4°C. Beads were then washed twice with the Dig-Wash
864 buffer, resuspended in the same buffer containing 2 mM CaCl₂, vortexed, and placed
865 on ice as soon as possible. After 30 min, a 2x stop buffer was added to quench the
866 digested reaction, and it was incubated at 37°C for 30 min. The suspensions were
867 collected and the DNA was extracted. We prepared the library using a NEBNext Ultra
868 II DNA library Prep Kit for Illumina (E7645, NEB), and sequenced the qualified
869 libraries with an Illumina Novaseq 6000 system to obtain paired-end 150-nt reads.

870 **ATAC-seq**

871 Zygote spermatocytes were collected by FACS as described in CUT&RUN, and
872 cells were washed twice with cold PBS. To prepare nuclei, cells were lysed with cold
873 lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM MgCl₂, and 0.1 % NP-
874 40), maintained on ice for 10 min, and centrifuged at 500 x g for 5 min at 4°C. After
875 carefully removing the suspension, we resuspended the pellet in the transposase
876 reaction mix (TD501, Vazyme) and incubated it at 37°C for 10 min. Fragments were
877 then immediately purified with 2 x AMPure XP beads (A63881, Beckman), and
878 library amplification was performed using the TruePrep DNA Library Prep Kit V2 for
879 Illumina (TD501, Vazyme).

880 **ChromHMM analyses**

881 Chromatin-state discovery and genome annotation with ChromHMM was carried

882 out by following the protocol by Ernst and Kellis (50) using ChromHMM software
883 v1.22. Based on the study by Spruce et al. (7) and personal communications with the
884 corresponding author Dr. Christopher L. Baker, we compiled the cellmarkfiletable
885 shown in Table S6. Datasets indicated in the Table were downloaded from GEO and
886 mapped to the mouse genome (mm10) using Bowtie2, and Bam files from sample
887 replicates were merged and binarized. The initial state map we created was compared
888 with the published version, and the correspondences between states in our initial map
889 and the one published (Table 1A by Spruce et al. (7)) were established visually; the
890 states in our initial map were then re-ordered to generate the final map by using the
891 “java -mx4000M -jar ChromHMM.jar Reorder” command. Data for other markers
892 that were either published or produced in the present study were aligned to the map by
893 using the “java -mx4000M -jar ChromHMM.jar OverlapEnrichment” command.

894 **RNA-seq analysis**

895 Our RNA-seq analyses followed a standard procedure that included mapping
896 sequence reads to the mouse genome mm10 by using Bowtie2 and identifying
897 differentially expressed genes by using the DESeq2 R package.

898 **ChIP-seq and CUT&RUN analysis**

899 ChIP-seq raw reads were trimmed to remove the adapter sequence when converting to
900 a fastq file, and the trimmed ChIP-seq reads were mapped to the UCSC mm10
901 genome using Bowtie2 (v2.4.1) (92) with default parameters. For BEND2, peak
902 calling was performed using Pepr (93) with default parameters and the corresponding
903 inputs as background, and peaks that mapped to blacklist (94) regions were removed.
904 For ADNP, CHD4, and ZMYM2, peak calling was performed using MACS2
905 (v2.2.7.1) (95) (<https://github.com/macs3-project/MACS>) with default parameters,
906 except that the q value was less than 0.01. Annotation of genomic locations and repeat
907 types were generated using HOMER (v4.11), and heatmaps were generated using the
908 command-line version of deepTools (v3.5.0) (96). Distribution of BEND2-binding
909 sites on the chromosomes were generated by CHIPseeker (v1.24.0) (97), and HOMER

910 (v4.11) was used with default settings to identify enriched motifs in BEND2 peaks.
911 RepeatMasker and TSS-location files were downloaded from the UCSC website, and
912 we achieved a Genome Browser view of the NGS data by using the command-line
913 version of pyGenomeTracks.

914 **ATAC-seq analysis**

915 Paired-end reads were aligned with Bowtie2 using default parameters, and only
916 uniquely mapping reads were retained for further analysis; PCR duplicates and
917 blacklist-region reads were removed. Peak calling was executed using MACS2
918 (v2.2.7.1). Different gene clusters and heatmaps were then generated using the
919 command-line version of deepTools (v3.5.0), and correlation analysis between the
920 three clusters of genes by ATAC-seq and differentially expressed genes was based
921 upon a hypergeometric distribution.

922 **Luciferase assay**

923 *Bend2* cDNA was cloned into a pFLAG-CMV-4 vector, and the BEND2-targeted
924 regions for *Dmrt1*, *Suz12*, *Lin28a*, and *Exo1* were PCR-amplified from mouse
925 genomic DNAs isolated from mouse tail tips and cloned into a PGL4.23-luciferase
926 vector (Promega, E8411). Five copies of a GGAAA sequence were synthesized by
927 Sangon Biotech and cloned into a PGL4.23-luciferase vector. TF-expressing plasmids,
928 promoter-luciferase plasmids, and the pRL-TK-*Renilla* constructs as internal controls
929 were co-transfected into 293FT cells on 96-well plates using X-Transcell reagent
930 (bjyf-Bio technology) following the manufacturer's protocol. Cell extracts were
931 prepared 48 h after transfection using the lysis buffer provided in the Dual-Luciferase
932 Reporter Assay System kit (Promega), and luciferase activity was measured on a
933 Synergy Neo2 Multi-Mode Microplate Reader instrument (Bio-Tek) according to the
934 manufacturer's protocol. *Renilla* luciferase activity was used to normalize the firefly
935 luciferase activity.

936 **Statistical analysis**

937 All experiments reported herein were independently repeated at least three times, and
938 all values in the Figures are depicted as mean \pm SEM unless stated otherwise. We used
939 Excel 2016 or GraphPad Prism 7 to perform statistical analyses. To analyze the
940 differences between two groups, we used two-tailed unpaired Student's *t*-tests. To
941 examine whether a group of genes (or genomic features) classified upon one
942 parameter were enriched with a group of genes classified upon another parameter, we
943 executed the R function phyper ($k-1$, M , $N-M$, n , lower.tail =FALSE), where N was
944 the total number of genes, M was the number of genes that were positive for the
945 second parameter, n was the number of genes that were positive for the first
946 parameter, and k was the number of genes that were positive for both parameters. This
947 function was based on a hypergeometric distribution. For statistical analysis of focus
948 number comparisons of RPA2, DMC1, and RAD51, data were analyzed with the
949 Tukey multiple-comparison test after one-way ANOVA. For statistical analysis of
950 inter-REC8 distance comparisons, *p* value was obtained with two-tailed, unpaired *t*-
951 test. No samples or animals were excluded from analyses, sample-size estimates were
952 not used, and the mice analyzed were litter mates. Investigators were not blinded to
953 mouse genotypes or cell genotypes during experiments. For all figures, *, **, and ***
954 represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. NS (not significant) indicates
955 not statistically significant (i.e., $p > 0.05$).

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Figures

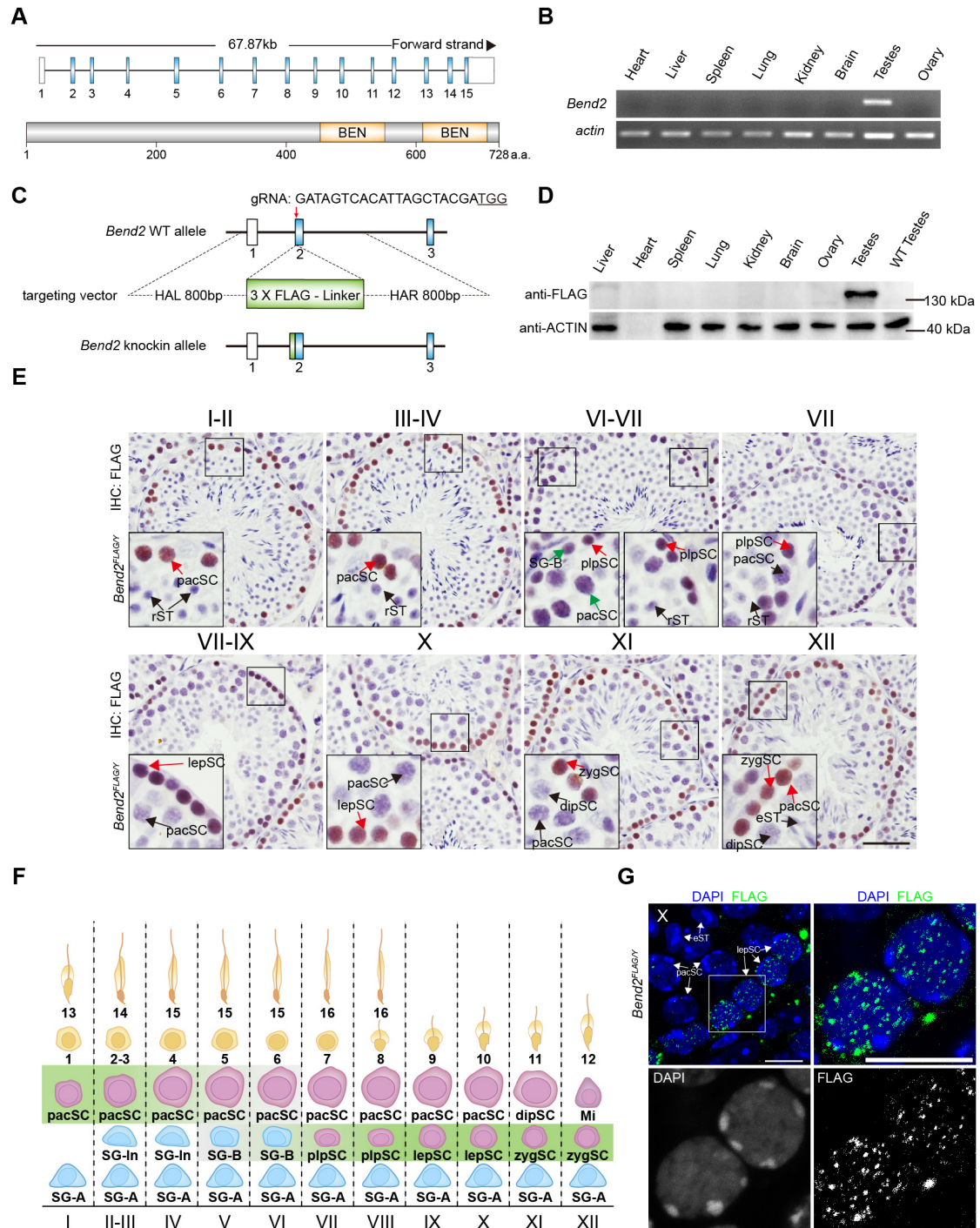


Fig. 1 BEND2 is a novel protein specifically expressed around the time of meiotic initiation.

(A) Schematic diagram of the primary structures of the *Bend2* gene and BEND2 protein. Upper diagram shows the structure of the *Bend2* gene; blue and white

rectangles indicate protein-coded exons and UTR regions, respectively. The lower diagram represents the BEND2 protein, with orange boxes indicating the BEN domain. **(B)** RT-PCR detection of *Bend2* expression in multiple mouse organs. **(C)** Schematic representation of the locus of the 3 x FLAG tag knock-in; the tag sequence was inserted immediately behind the first codon of BEND2. **(D)** Western blotting analyses of BEND2 expression in multiple mouse organs using mmAb-FLAG. **(E)** Immunohistochemical staining of FLAG-BEND2 in testicular sections of various seminiferous stages using mmAb-FLAG; red and green arrows indicate BEND2-strongly- and -weakly-expressing cells, respectively; black arrows indicate no BEND2 expression. SG-B, type B spermatogonia; plpSC, pre-leptotene spermatocytes; lepSC, leptotene spermatocytes; zygSC, zygotene spermatids; pacSC, pachytene spermatocytes; dipSC, diplotene spermatocytes; rST, round spermatids; eST, elongated spermatids (scale bar, 20 μm). **(F)** Schematic summary of FLAG-BEND2 expression in male germ cell types and seminiferous stages. **(G)** Immunofluorescence staining of FLAG-BEND2 shown at higher magnification of BEND2 signals in leptotene spermatocytes (scale bar, 10 μm).

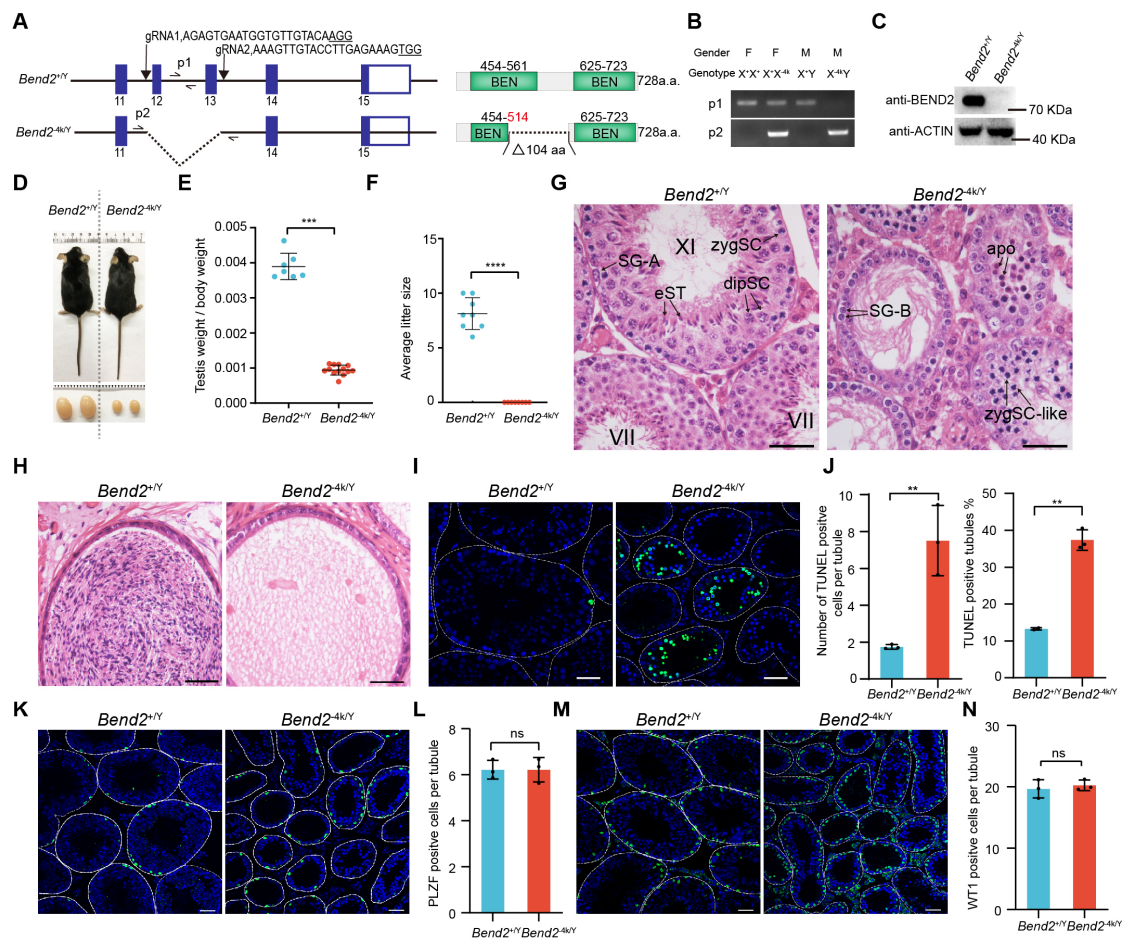


Fig. 2 BEND2 is required for mouse spermatogenesis and the maintenance of male fertility.

(A) Schematic illustration of the deletion of two exons of the *Bend2* gene to generate *Bend2*^{4k/Y} mice; p1 and p2 indicate that two primers were used in mouse genetic identification. The right diagram shows BEND2 protein structure in WT and KO mice. (B) Identification of genotype with p1 (wild type allele) and p2 (mutant allele) primer pairs. (C) Western blot confirmation of the elimination of BEND2 protein in *Bend2*^{4k/Y} mice using rpAb-B2. (D) Note the significant size reduction in 8-week-old *Bend2*^{4k/Y} testes. (E) Quantitative comparison of testis/body ratios between *Bend2*^{+/Y} and *Bend2*^{4k/Y} mice (***p<0.001, Student's t-test). (F) Comparison of litter size in *Bend2*^{+/Y} and *Bend2*^{4k/Y} mice (****p<0.0001). (G) H&E staining of testicular sections in *Bend2*^{+/Y} and *Bend2*^{4k/Y} mice. zygSC-like, zygotene-like spermatocytes; apo, apoptotic cells (scale bar, 20 μm). (H) H&E staining of epididymal sections of *Bend2*^{+/Y} and *Bend2*^{4k/Y} mice. (I) TUNEL staining of testicular sections in *Bend2*^{+/Y}

and *Bend2*^{-4k/Y} mice; green signals indicate apoptotic cells (scale bar, 50 μ m). **(J)** Quantitative comparison of TUNEL staining shows both TUNEL-positive cells and tubules were increased in *Bend2*^{-4k/Y} mice. **(K, L)** Immunofluorescence staining indicates that the number of PLZF-positive cells (green) were the same between *Bend2*^{+Y} and *Bend2*^{-4k/Y} mice (scale bar, 20 μ m). **(M, N)** Quantitative comparison of WT1 immunofluorescence staining of testicular sections in *Bend2*^{+Y} and *Bend2*^{-4k/Y} mice (scale bar, 20 μ m).

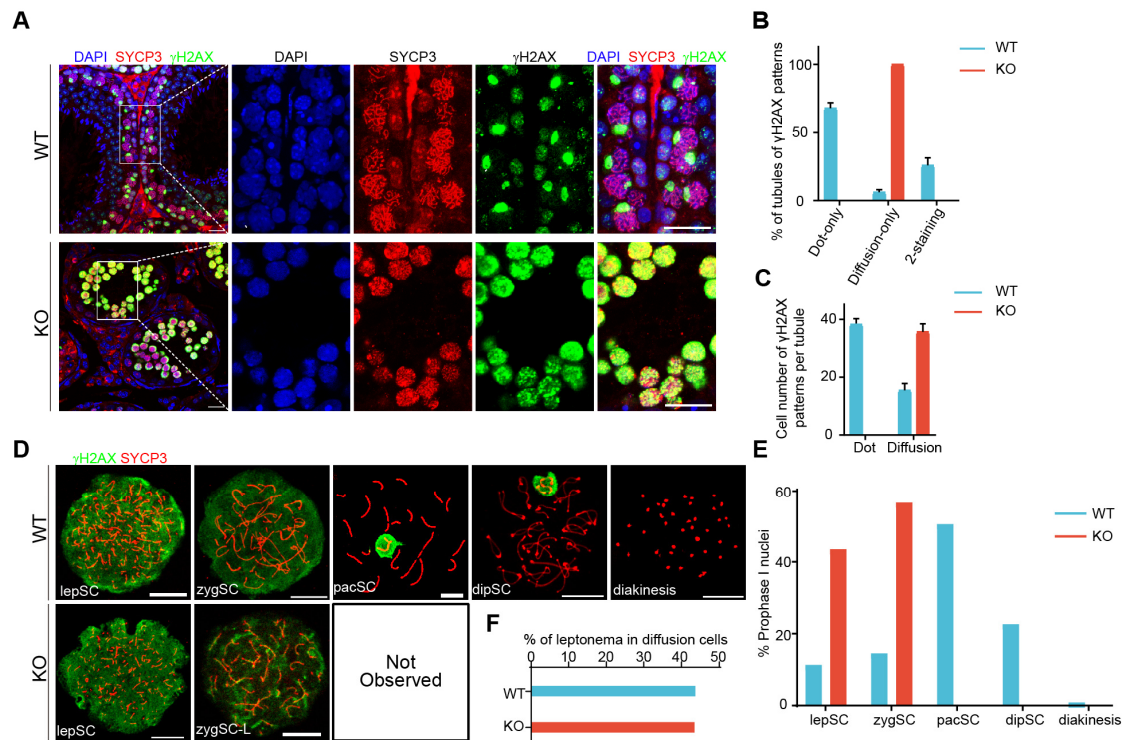


Fig. 3 Spermatocytes with *Bend2* knockout fail to complete meiotic prophase.

(A) Immunofluorescent labeling of testicular sections with mouse polyclonal SYCP3 antibodies (red) and rat polyclonal γ H2AX antibodies (green). DNA was counterstained with DAPI (blue) and merged images are shown (scale bar, 10 μ m).

(B) Proportion of tubules with γ H2AX expression patterns. Tubules of each mouse were counted: for *Bend2*^{+Y}, n= 354; for *Bend2*^{-4k/Y}, n=321. **(C)** Average number of spermatocytes with γ H2AX expression patterns per tubule. At least 100 tubules of each mouse were counted.

(D) Nuclear spreads of various spermatocytes in *Bend2*^{+Y} and *Bend2*^{-4k/Y} mice. Spermatocytes were immunostained with SYCP3 (red) and γ H2AX (green) (scale bar, 10 μ m). **(E)** Frequency statistics for spermatocytes in the meiotic-prophase I stage for WT and KO mice. Number of spermatocytes analyzed: for *Bend2*^{+Y}, n= 256; for *Bend2*^{-4k/Y}, n=180. **(F)** Proportions of leptonema in γ H2AX diffusion cells.

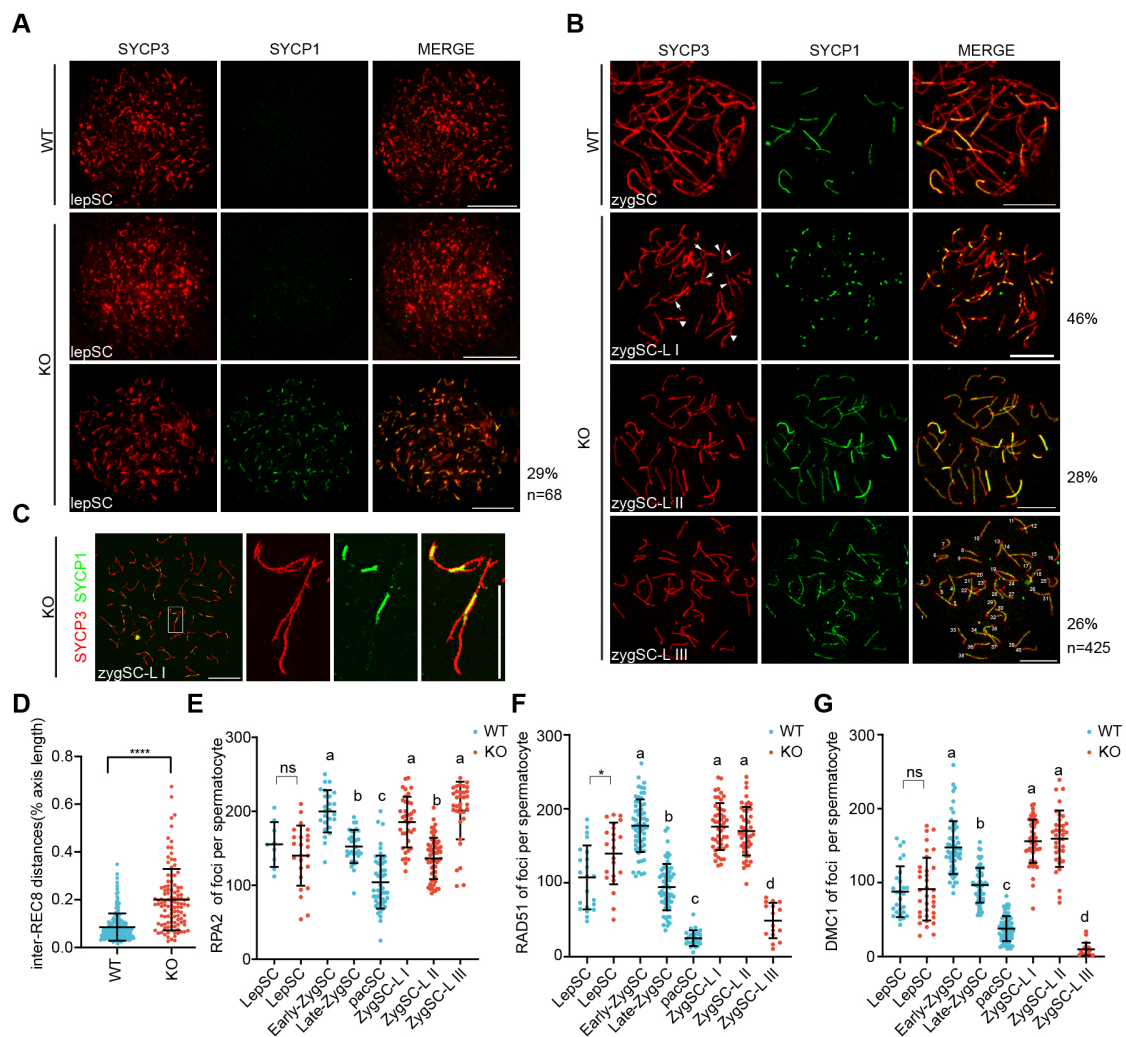


Fig. 4 BEND2 is required for homologous synapsis in meiosis.

(A-B) Immunofluorescent labeling of SYCP3 (red) and the transverse filament protein SYCP1 (green), a marker of synapsis. **(A)** Abnormal SYCP1 signals were observed in leptotene spermatocytes of *Bend2*^{4k/Y} mice compared with *Bend2*^{+Y} mice. Approximately 30% of leptotene spermatocytes were abnormal in *Bend2*^{4k/Y} mice (n=68). **(B)** SYCP3- and SYCP1-staining of *Bend2*^{+Y} zygote spermatocytes and *Bend2*^{4k/Y} zygote-like spermatocytes. According to their staining with SYCP1 and SYCP3, zygote-like spermatocytes were divided into three classes: zygSC-L I, 46%; zygSC-L II, 28%; and zygSC-L III, 26% (n=425). **(C)** Super-resolution microscopic images of zygote-like spermatocytes showing abnormal synapsis (scale bar, 10 μ m). **(D)** Scatterplot in which we compared inter-REC8 distances along chromosomes in *Bend2*^{+Y} and *Bend2*^{4k/Y} mice ($p < 0.0001$, obtained with two-tailed, unpaired *t*-test). **(E)** Each dot represents the number of RPA2 foci per spermatocyte; solid lines show the

mean and SD of focus number in each group of spermatocytes. Data were analyzed with one-way ANOVA and Tukey's multiple-comparison test. **(F)** Each dot represents the number of RAD51 foci per spermatocyte. Solid lines show the mean and SD of focus number in each group of spermatocytes. Data were analyzed with one-way ANOVA and Tukey's multiple comparison test. **(G)** Each dot represents the number of DMC1 foci per spermatocyte. Solid lines show the mean and SD of focus number in each group of spermatocytes. Data were analyzed with one-way ANOVA followed by Tukey's multiple comparison test.

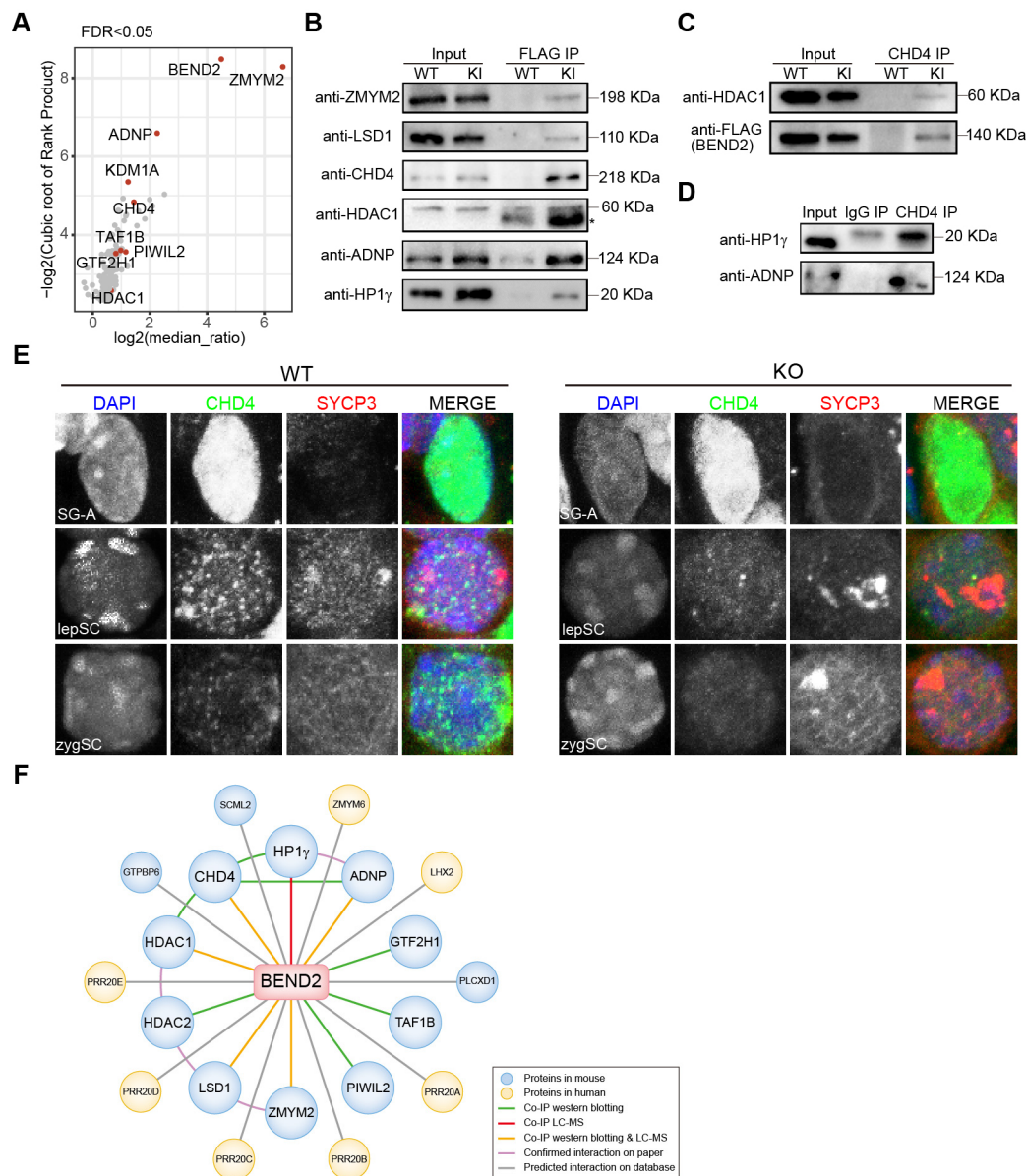


Fig. 5 MS analyses of BEND2-interacting factors in testicular extracts.

(A) LC-MS/MS analysis of enriched protein from co-IP. Protein identification was performed in the presence of wash buffer containing 300 mM or 500 mM NaCl; WT mice served as background controls (n=3 independent biological replicates, with each replicate containing two 15-dpp KI mice). **(B)** Co-immunoprecipitation of BEND2 with ZMYM2, LSD1, CHD4, HDAC1, ADNP, and HP1 γ in testis from WT and *Bend2*^{FLAG/Y} mice at 15 dpp. **(C)** Co-IP western blotting analysis used to confirm the interaction of CHD4 and BEND2 or HDAC1 in the testis. **(D)** Co-IP western blotting analysis to confirm the interaction of CHD4 and HP1 γ or ADNP in the testis. **(E)** Immunofluorescence of testicular sections with SYCP3 (red) and CHD4 (green) in

WT and KO mice, and DNA was counterstained with DAPI (blue); their merged images are shown (scale bar, 10 μ m). **(F)** The network of BEND2-interacting proteins.

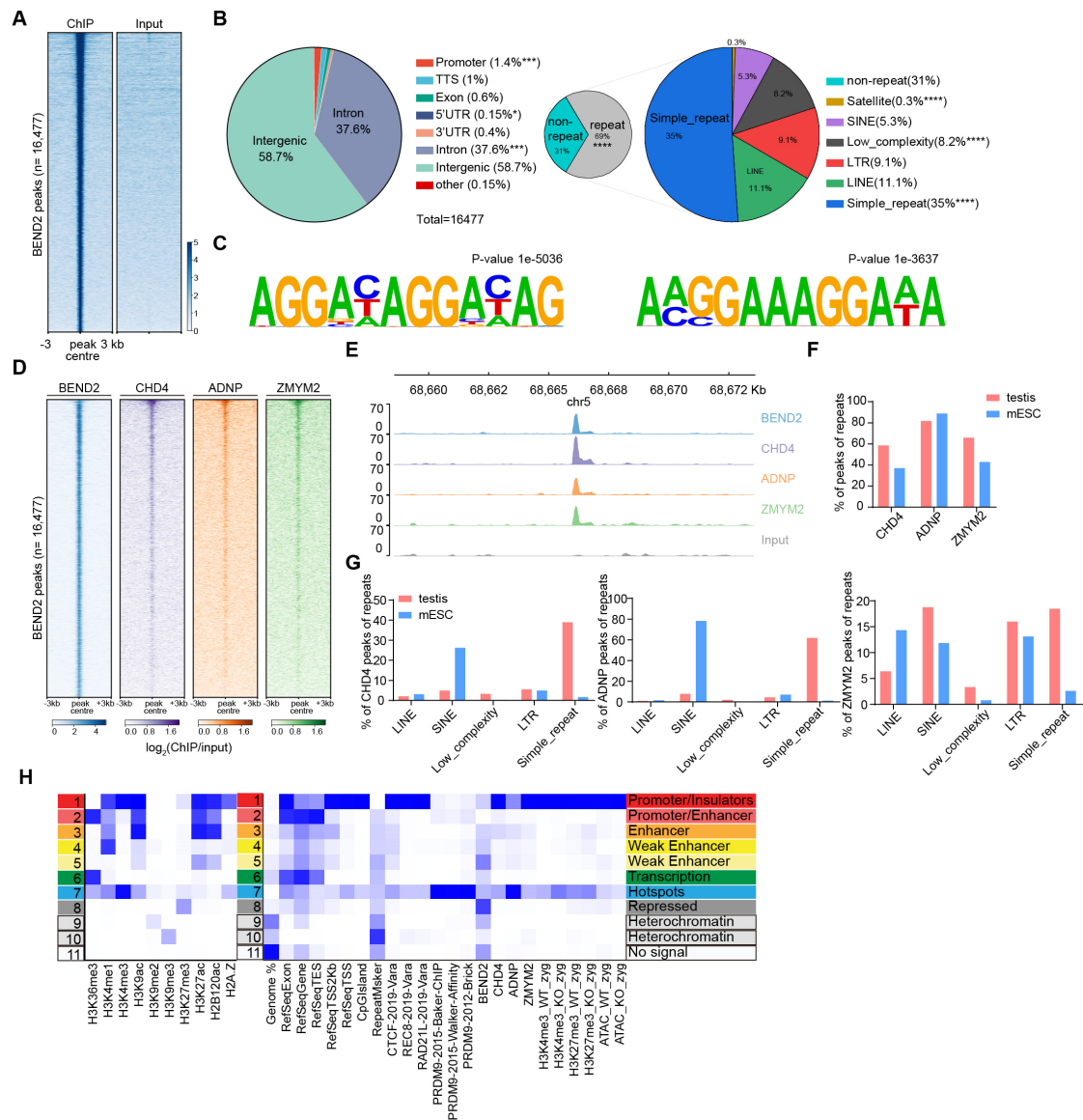


Fig. 6 BEND2 binds to multiple chromatin states

(A) Heatmap of BEND2 ChIP-seq enrichment across all significant peaks (n=16,477) in the mouse genome. Each row represents a 6-kb window centered on BEND2 peak midpoints, sorted by the BEND2 ChIP signal. Input signals at the same position are shown on the right (average peak intensity of n=6 biological replicates). **(B)** BEND2-binding sites were classified by their genomic locations and repeat types as indicated. **(C)** The top BEND2 DNA-binding motif predicted by HOMER (left); sequences with the top motif re-analyzed by HOMER (right). **(D)** Heatmap of BEND2, CHD4, ADNP, and ZMYM2 ChIP-seq enrichment across all BEND2 peak midpoints; each raw datum represents a 6-kb window centered on BEND2 peak midpoints. **(E)** Browser view showing ChIP-seq signals of BEND2, CHD4, ADNP, and ZMYM2 co-binding sites. **(F)**

Comparison of repeat percentages for CHD4, ADNP, and ZMYM2 in testis and ESC.

(G) Enrichment comparison of different repeat classes peaks in testis and ESC with respect to CHD4, ADNP, and ZMYM2. **(H)** Heatmap of chromatin states produced by ChromHMM based on 10 histone modifications (left); heatmap showing different enrichment for indicated annotations for each state (right).

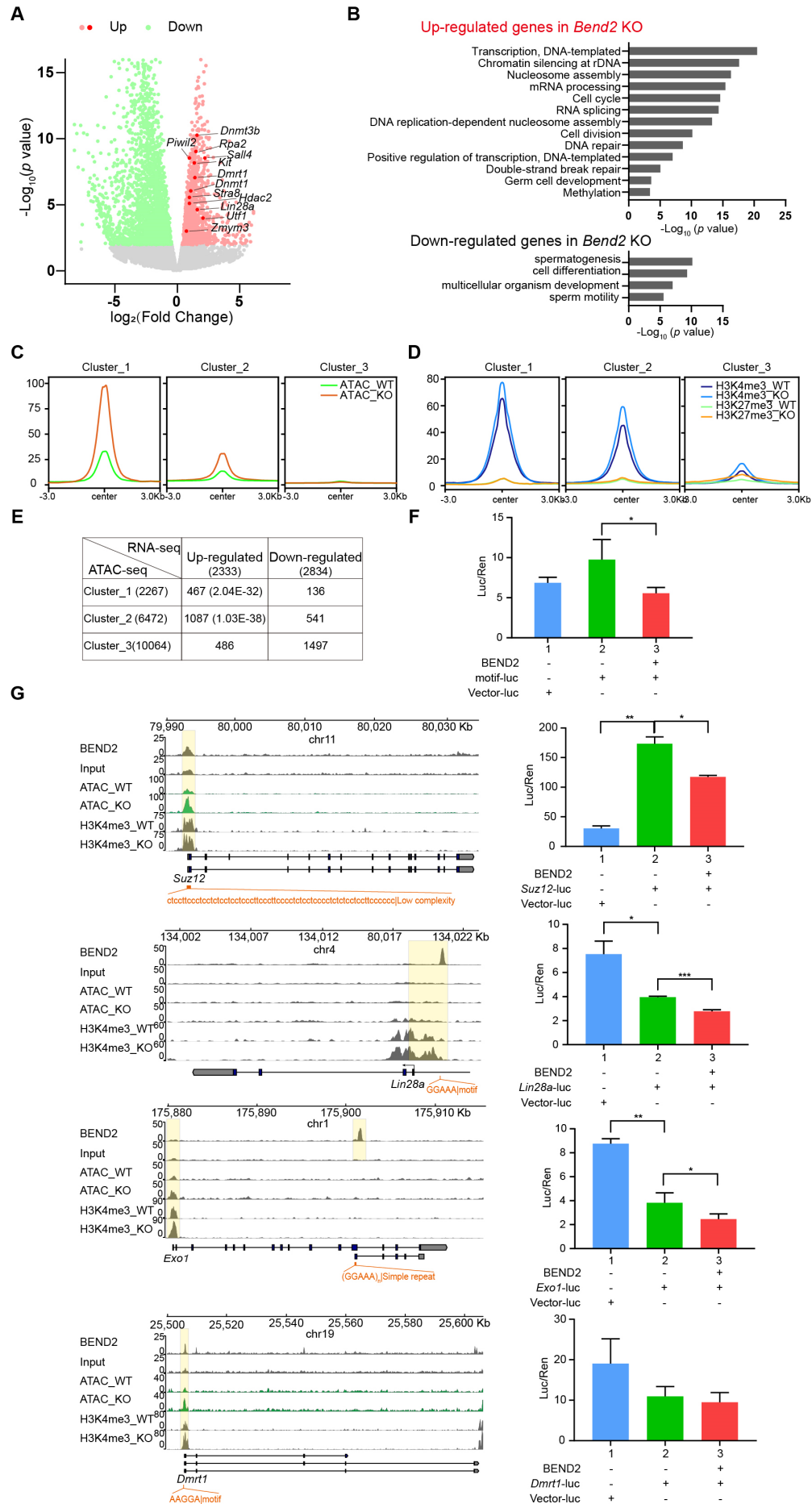


Fig. 7 Transcriptomic and epigenetic states change after *Bend2* knockout.

(A) Volcano plot of transcript levels between cells from adult WT and *Bend2*^{4k/Y} mice using lepSCs and zygSCs. The differentially expressed genes are highlighted in red (upregulated in *Bend2*^{4k/Y}) and green (downregulated in *Bend2*^{4k/Y}). **(B)** Representative Gene Ontology (GO) terms of the biological process categories enriched in differentially expressed genes. **(C)** Average distribution of ATAC-seq signal around the TSS of three clusters of genes. **(D)** Average distribution of H3K4me3 and H3K27me3 around the TSS of three clusters of genes. **(E)** Correlation analysis between the three clusters of genes by ATAC-seq and differentially expressed genes. **(F)** Validation of DNA-binding motif of BEND2 using dual-luciferase assay. **p*<0.05 **(G)** Browser view showing BEND2 ChIP-seq, ATAC-seq, and H3K4me3 ChIP-seq signals of target genes (left). Dual-luciferase assay showing the repression of BEND2 target genes (n=3, ***p*<0.01 [right]).

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