



HAL
open science

Mouse ANKRD31 Regulates Spatiotemporal Patterning of Meiotic Recombination Initiation and Ensures Recombination between X and Y Sex Chromosomes

Frantzeskos Papanikos, Julie a J Clément, Erika Testa, Ramya Ravindranathan, Corinne Grey, Ihsan Dereli, Anastasiia Bondarieva, Sarai Valerio-Cabrera, Marcello Stanzione, Alexander Schleiffer, et al.

► To cite this version:

Frantzeskos Papanikos, Julie a J Clément, Erika Testa, Ramya Ravindranathan, Corinne Grey, et al.. Mouse ANKRD31 Regulates Spatiotemporal Patterning of Meiotic Recombination Initiation and Ensures Recombination between X and Y Sex Chromosomes. *Molecular Cell*, 2019, 74 (5), pp.1069-1085.e11. <10.1016/j.molcel.2019.03.022>. <hal-02359134>

HAL Id: hal-02359134

<https://hal.science/hal-02359134v1>

Submitted on 25 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC 4.0 - Attribution - Non-commercial use - International License

1 **ANKRD31 regulates spatiotemporal patterning of meiotic recombination initiation and**
2 **ensures recombination between heterologous sex chromosomes in mice**

3 **Authors**

4 Frantzeskos Papanikos^{1‡}, Julie A.J. Clément^{2‡}, Erika Testa³, Ramya Ravindranathan¹, Corinne
5 Grey², Ihsan Dereli¹, Anastasiia Bondarieva¹, Sarai Valerio-Cabrera¹, Marcello Stanzione^{1,9},
6 Alexander Schleiffer^{5,6}, Petr Jansa⁴, Diana Lustyk⁴, Ji-Feng Fei⁷, Ian R. Adams⁸, Jiri Forejt⁴,
7 Marco Barchi³, Bernard de Massy^{2*}, Attila Toth^{1*†}

8 **Affiliations**

9 ¹ Institute of Physiological Chemistry, Faculty of Medicine, Technische Universität Dresden,
10 Fetscherstraße 74, 01307 Dresden, Germany

11 ² Institute of Human Genetics, UMR 9002, CNRS, Université de Montpellier, 34396 Montpellier
12 cedex 5, France

13 ³ Department Biomedicine and Prevention, Faculty of Medicine, University of Rome Tor Vergata,
14 Via Montpellier n.1, 00133, Rome, Italy

15 ⁴ Institute of Molecular Genetics, Division BIOCEV, Prumyslova 595, 25250 Vestec, Czech
16 Republic

17 ⁵ Research Institute of Molecular Pathology (IMP), Campus-Vienna-Biocenter 1, Vienna
18 BioCenter (VBC), 1030 Vienna, Austria

19 ⁶ Institute of Molecular Biotechnology (IMBA), Dr. Bohr-Gasse 3, Vienna BioCenter (VBC), 1030
20 Vienna, Austria

21 ⁷ Institute for Brain Research and Rehabilitation, South China Normal University, 510631,
22 Guangzhou, China

23 ⁸ MRC Human Genetics Unit, MRC Institute of Genetics and Molecular Medicine, University of
24 Edinburgh, Western General Hospital, Edinburgh, EH4 2XU, United Kingdom

25 ⁹ Current address: Massachusetts General Hospital Cancer Center, Harvard Medical School,
26 Charlestown, Massachusetts 02129, USA

27

28 E-mails:

29 frantzeskos.papanikos@mailbox.tu-dresden.de

30 julie.clement@igh.cnrs.fr
31 corinne.grey@igh.cnrs.fr
32 erikatesta87@gmail.com
33 ihsan.dereli@mailbox.tu-dresden.de
34 ramya.ravindranathan@tu-dresden.de
35 Anastasiia.Bondarieva@mailbox.tu-dresden.de
36 sarai.valerio_cabrera@tu-dresden.de
37 MSTANZIONE@mgh.harvard.edu
38 pjansa@img.cas.cz
39 diana.lustyk@img.cas.cz
40 jiri.forejt@img.cas.cz
41 Alexander.Schleiffer@imp.ac.at
42 jifeng.fe@imp.ac.at
43 Ian.Adams@igmm.ed.ac.uk
44 marco.barchi@uniroma2.it
45 bernard.de-massy@igh.cnrs.fr
46 attila.toth@mailbox.tu-dresden.de
47 [‡] Equal contribution to work
48 ^{*} Correspondence to bernard.de-massy@igh.cnrs.fr and attila.toth@mailbox.tu-dresden.de
49 ^{††} Lead author
50

51 **Summary**

52 Orderly segregation of chromosomes during meiosis requires that crossovers form between
53 homologous chromosomes by recombination. Programmed DNA double-strand breaks (DSBs)
54 initiate meiotic recombination. We identify ANKRD31 as a key component of complexes of DSB-
55 promoting proteins which assemble on meiotic chromosome axes. Genome-wide, ANKRD31
56 deficiency causes delayed recombination initiation. In addition, loss of ANKRD31 alters DSB
57 distribution owing to reduced selectivity for sites that normally attract DSBs. Strikingly,
58 ANKRD31 deficiency also abolishes uniquely high rates of recombination that normally
59 characterize pseudoautosomal regions (PARs) of X and Y chromosomes. Consequently, sex
60 chromosomes do not form crossovers leading to chromosome segregation failure in ANKRD31-
61 deficient spermatocytes. These defects co-occur with a genome-wide delay in assembling DSB-
62 promoting proteins on autosome axes and a loss of a specialized PAR-axis domain that is highly
63 enriched for DSB-promoting proteins in wild-type. Thus, we propose a model for spatiotemporal

64 patterning of recombination by ANKRD31-dependent control of axis-associated DSB-promoting
65 proteins.

66

67 **Introduction**

68 Programmed DNA double-strand breaks (DSBs) are critical for meiosis, as they initiate meiotic
69 homologous recombination, and their repair generates reciprocal DNA exchanges, called
70 crossovers (CO). In most organisms, COs must form between each pair of homologous
71 chromosomes (homologs) for correct chromosome segregation in the first meiotic division
72 (Hunter, 2015). Due to the importance and the potential genotoxicity of DSBs, DSB formation
73 and repair are under tight spatiotemporal control (Keeney et al., 2014).

74 Meiotic DSB formation and repair take place within the context of the meiosis-specific
75 chromosome axis and the synaptonemal complex (SC), reviewed in (de Massy, 2013; Hunter,
76 2015; Keeney et al., 2014). Initially, multiple DSBs are formed along the axes, which are linear
77 chromatin structures along the shared core of sister chromatid pairs in meiotic prophase. Single-
78 stranded DNA ends formed by processing of DSBs invade homologs, which promotes alignment
79 and synapsis of homolog axes. It is the resultant SC where recombination completion, including
80 CO formation, occurs.

81 DSB formation is catalyzed by an evolutionary conserved topoisomerase-like enzyme complex,
82 consisting of the SPO11 enzyme and its binding partner TOPOVIBL (Bergerat et al., 1997;
83 Keeney et al., 1997; Robert et al., 2016; Vrielynck et al., 2016). SPO11 activity depends on a
84 partially conserved set of auxiliary proteins, MEI4, REC114 and IHO1 in mice, which assemble
85 into axis-bound complexes, called pre-DSB recombinosomes, prior to DSB formation (de Massy,
86 2013; Kumar et al., 2010; Stanzione et al., 2016). Pre-DSB recombinosomes are thought to
87 promote DSBs by activating SPO11 on axes. Anchoring of pre-DSB recombinosomes to axes
88 relies on IHO1 (Mer2 in budding yeast) and meiosis-specific HORMA-domain proteins in both
89 budding yeast and mammals (Kumar et al., 2015; Kumar et al., 2018; Panizza et al., 2011;
90 Stanzione et al., 2016). HORMAD1-IHO1 assemblies can form independent of MEI4 or REC114
91 thereby providing an axis-bound platform for pre-DSB recombinosome assembly (Kumar et al.,
92 2018; Stanzione et al., 2016). HORMAD1, IHO1 and pre-DSB recombinosomes are restricted to
93 unsynapsed axes, which is hypothesized to concentrate DSB forming activity to genomic regions
94 requiring DSBs for completion of homolog alignment (Kauppi et al., 2013; Stanzione et al., 2016;
95 Wojtasz et al., 2009).

96 Meiotic chromatin is thought to be arrayed into loops anchored on chromosome axes (Zickler
97 and Kleckner, 1999) and DSBs seem to form in these loops (Blat et al., 2002; Panizza et al.,
98 2011). Sites of frequent DSB formation, called hotspots, are also associated with open
99 chromatin marks, such as trimethylation of histone H3 lysine 4 (H3K4me3) and lysine 36
100 (H3K36me3) (Buard et al., 2009; Davies et al., 2016; Grey et al., 2017; Powers et al., 2016;
101 Smagulova et al., 2011). Thus, axis-bound pre-DSB recombinosomes are hypothesized to target
102 SPO11 activity by recruiting open chromatin sites from within loops to the axis via a poorly
103 understood mechanism, reviewed in (Grey et al., 2018). In most mammals, DSB hotspots
104 emerge at sites bound by the histone methyltransferase PRDM9 in a sequence specific manner.
105 PRDM9 generates the histone marks H3K4me3 and H3K36me3 at these sites (Grey et al.,
106 2018). Additionally, by an unknown mechanism, PRDM9 prevents DSBs at other H3K4me3-rich
107 genomic locations such as transcription start sites or enhancers, which serve as hotspots in
108 *Prdm9*^{-/-} meocytes (hereafter called default hotspots) (Brick et al., 2012; Diagouraga et al.,
109 2018). An exception to this rule is the pseudoautosomal region (PAR) of X and Y chromosomes,
110 where high DSB activity occurs independently of PRDM9 (Brick et al., 2012). X and Y
111 chromosomes are homologous only in the PAR, therefore at least one CO must form in this
112 relatively short region to ensure segregation of these chromosomes. Thus, DSB density is
113 between 10 to 110 fold higher in the PAR than the rest of the genome (Kauppi et al., 2011;
114 Lange et al., 2016). High DSB activity is possibly enabled by a unique PAR-chromatin
115 environment characterized by both strong accumulation of PRDM9-independent histone
116 H3K4me3 (Brick et al., 2012), and the combination of a disproportionately long axis and short
117 chromatin loops (Kauppi et al., 2011). However, the importance of these features in DSB control
118 has not been tested, and mechanisms underlying high recombination rate in the PAR remain
119 enigmatic.

120 Here we report the identification of a novel meiotic protein, ANKRD31, which plays a
121 major role in the temporal and spatial control of meiotic DSBs. Strikingly, ANKRD31 is required
122 to prevent DSBs at default hotspot sites, and to ensure DSBs/COs in the PAR. Both roles likely
123 rely on ANKRD31's function in organizing axis-bound pre-DSB recombinosomes.

124

125 **Results**

126 **Conserved ANKRD31 associates with pre-DSB recombinosomes**

127 To identify new players in meiotic recombination we searched for genes that are preferentially
128 expressed in mouse gonads where prophase meocytes are present. We reviewed ENCODE

129 transcriptome datasets (Consortium, 2011, 2012) and profiled expression in reproductive
130 tissues. This approach identified a previously uncharacterized gene, *Ankrd31* (Figure 1A, 1B and
131 1SA). *Ankrd31* encodes a 1857 amino acid protein (XP_006517860) that is conserved among
132 vertebrates (Figure 2S). In most vertebrate taxa, ANKRD31 contains two separated triplets of
133 Ankyrin repeats, which are common protein-protein interaction motifs (Mosavi et al., 2004). C-
134 terminal to the Ankyrin repeats there are three further conserved domains, a predicted coiled-coil
135 domain and two regions without functional predictions.

136 Immunostaining of histological sections of testes reconfirmed presence of ANKRD31 in meiotic
137 cells (Figure 1C and S1B). Antibodies to both an N- and a C-terminal fragment of ANKRD31
138 stained spermatocyte nuclei from premeiotic DNA replication until early pachytene. In contrast,
139 somatic cells lacked a consistent ANKRD31 signal. Localization of ANKRD31 was analyzed in
140 detail in nuclear surface spread spermatocytes at distinct prophase sub-stages (Figure 1D and
141 Table S1, see STAR Methods for staging prophase). Focal ANKRD31 staining was detected on
142 chromatin between preleptotene and early pachytene with focus numbers peaking in leptotene
143 (Figure 1D-G and S1C-F). Most ANKRD31 foci associated with the chromosome axes (85.5% in
144 leptotene, n=29, and 88.7% in early zygotene, n=32, spermatocytes), but foci were depleted
145 from axes upon SC formation (Figure 1E, S1C and S1E). Beyond numerous relatively small foci
146 (median diameter, 0.26 μm), ANKRD31 also formed up to eight larger aggregates (median
147 diameter 0.62 μm) from preleptotene to pachytene (Figure 1D, 1F and S1F). Median numbers of
148 aggregates peaked at five in leptotene and declined to three as spermatocytes progressed to
149 early pachytene (n=108 spermatocytes). ANKRD31 localization resembled the localization of
150 two pre-DSB recombinosome components, MEI4 and REC114 (Kumar et al., 2010; Kumar et al.,
151 2015; Stanzione et al., 2016). We found substantial colocalization between ANKRD31 and MEI4
152 or REC114 foci (Figure 2A-F, S3A), and large ANKRD31 aggregates always colocalized with
153 similar aggregates of REC114 (n=98 spermatocytes) and MEI4 (n=89 spermatocytes and n=24
154 oocytes, Figure 2A, 2D, 2G and 3SA-C) in both sexes.

155 Yeast two-hybrid (Y2H) assays also indicated that ANKRD31 directly interacted with two known
156 components of pre-DSB recombinosomes, IHO1 and REC114, but not with HORMAD1 (Figure
157 2H, S3D and Table S2), which acts as an anchor for pre-DSB recombinosomes on axes (Kumar
158 et al., 2015; Stanzione et al., 2016). These results suggest that ANKRD31 might be incorporated
159 into pre-DSB recombinosomes by directly interacting with REC114 and IHO1.

160

161 **ANKRD31 forms aggregates in PARs**

162 We examined ANKRD31 aggregates closer, because aggregates of pre-DSB recombinosome
163 components were poorly characterized before. In zygotene and pachytene, where chromosome
164 axes were well developed, it was apparent that aggregates of ANKRD31, REC114 and MEI4
165 associated with the ends of a subset of chromosomes (Figure 1D, 1F, 2G S3B and S3C). In
166 spermatocytes, one of the aggregates always associated with PARs of X and Y chromosomes.
167 The rest of the aggregates were associated with up to three non-centromeric autosomal ends
168 (Figure S1F). Consistent with these observations, an independent study by Acquaviva et al.
169 2019 discovered that the presence of similar sequences in PARs and the non-centromeric ends
170 of autosome 4, 9 and 13 is associated with ANKRD31 aggregate formation at these loci in both
171 oocytes and spermatocytes. Peculiarly, whereas ANKRD31, MEI4, and REC114 foci were
172 detectable only on unsynapsed axes, aggregates persisted on synapsed chromosome ends till
173 early pachytene, which was most obvious in PARs in spermatocytes (Figure 1D, 1F, 2G and
174 S3B-C). Aggregates disappeared only after meiosis progressed beyond early pachytene
175 (Figure 1D and not shown).

176 **Distinct molecular requirements for ANKRD31 aggregates and foci**

177 Given their differential persistence in synapsed regions, foci and aggregates of
178 ANKRD31/MEI4/REC114 might represent qualitatively different protein complexes with distinct
179 underlying molecular requirements. To test this possibility we compared localization of
180 ANKRD31, REC114 and MEI4 in *Mei4*^{-/-}, *Rec114*^{-/-} and *Iho1*^{-/-} spermatocytes (Figure 2J and
181 S3E-I). Previously, strongly reduced REC114 and MEI4 focus numbers were reported in *Mei4*^{-/-}
182 and *Rec114*^{-/-} mice, respectively (Kumar et al., 2018). In addition, we found that both focus and
183 aggregate formation of ANKRD31, REC114 and MEI4 were each disrupted in *Mei4*^{-/-} and
184 *Rec114*^{-/-} spermatocytes (Figure S3E-G and Table S3). Remarkably, only ANKRD31 (Figure 2I),
185 MEI4 and REC114 foci (Stanzione et al., 2016), but not aggregates, were diminished in *Iho1*^{-/-}
186 spermatocytes (Figure 2J). ANKRD31 aggregates formed efficiently in *Iho1*^{-/-} spermatocytes;
187 median numbers of aggregates were four in both wild type (n=62) and *Iho1*^{-/-} (n=57)
188 spermatocytes in zygotene. These aggregates always colocalized with aggregates of MEI4
189 (n=100) and REC114 (n=100) in *Iho1*^{-/-} spermatocytes (Figure 2J and S3H). ANKRD31
190 aggregates also colocalized with PAR FISH signals in late zygotene-like *Iho1*^{-/-} spermatocytes
191 (≥94% of FISH signals and ANKRD31 aggregates colocalize with each other, n=51) (Figure
192 S3I). These observations support the hypothesis that ANKRD31 interacts with MEI4/REC114
193 complexes. Furthermore, these data show that distinct molecular requirements underpin focus
194 formation of ANKRD31, MEI4 and REC114 in non-PAR regions and aggregate formation of the

195 same proteins in PARs and at PAR-like ends of chromosome 4, 9 and 13 (as identified by
196 (Acquaviva et al., 2019).

197 **Loss of ANKRD31 affects fertility of mice**

198 To test ANKRD31's functions we generated two mouse lines (by CRISPR/Cas9 editing) with
199 distinct frameshift mutations in exon 6 of *Ankrd31* (Figure 3A). These lines had indistinguishable
200 phenotypes in initial analysis, hence, we focused only on one of them, mut1. A frameshift after
201 the 134th codon in the longest open reading frame (ORF) of *Ankrd31*, is predicted to severely
202 truncate ANKRD31 in the mut1 line. Hence, we refer to mut1 as a loss of function allele
203 (*Ankrd31*^{-/-}) hereafter. Immunoprecipitation-western blot analysis of testis extracts and
204 immunofluorescence in fixed gonadal samples reconfirmed that ANKRD31 domains which are
205 C-terminal to the frameshift position are not produced in *Ankrd31*^{-/-} meiocytes (Figure S4A-F).
206 Disruption of *Ankrd31* caused no obvious somatic phenotypes but led to fertility defects.
207 Whereas female *Ankrd31*^{-/-} mice were fertile, they lost fertility faster than wild-type in advanced
208 age. Average numbers of pups/breeding week were 1.53 and 1.38 between 8-28 weeks of age
209 (not significant), and 0.92 and 0.45 beyond 28 weeks of age (p=0.0272, paired t-test) in wild-
210 type and *Ankrd31*^{-/-} mothers, respectively (n=6 mice for each genotype). Male *Ankrd31*^{-/-} mice
211 were infertile (no pups after 88 breeding weeks, n=5 males).

212 To test if these fertility defects were caused by a reduced capacity of *Ankrd31*^{-/-} meiocytes to
213 progress through meiosis, we examined oocytes and spermatocytes in histological sections.
214 Consistent with reduced female fertility, we observed 4.95 fold lower median oocyte numbers in
215 *Ankrd31*^{-/-} mice than wild-type at 6-7 weeks of age (Figure S4G). This reduction in oocyte
216 number was associated with elevated rates of apoptosis, as determined by cleaved PARP1
217 staining (Daniel et al., 2011; Ghafari et al., 2007), in the oocytes of newborn *Ankrd31*^{-/-} mice as
218 compared to wild-type (Figure S4H). Consistent with a loss of fertility in males, testis weight was
219 2.7 fold lower in *Ankrd31*^{-/-} than wild-type mice (Figure S4I). Investigation of gametogenesis in
220 histological sections revealed elevated apoptosis after mid pachytene in spermatocytes of
221 *Ankrd31*^{-/-} mice (Figure S4J and Table S4). Nevertheless, noticeable fractions of spermatocytes
222 progressed to the first meiotic metaphase, where they arrested, which was evidenced by the
223 elevated proportion of seminiferous tubules that contained metaphase cells (Figure 3B and 3C).
224 These metaphase cells often contained chromosomes that did not line up at the metaphase
225 plate (1.7% and 89% in n=173 and n=146 wild type and *Ankrd31*^{-/-} meiotic metaphase I
226 spermatocytes, respectively), which indicated chromosome alignment defects in *Ankrd31*^{-/-} mice
227 (Figure 3B). Most metaphase spermatocytes underwent apoptosis and only few post-meiotic
228 cells were detected in *Ankrd31*^{-/-} mice (Figure S4J).

229 We conclude that compromised meiocyte survival provides a plausible explanation for fertility
230 defects in *Ankrd31*^{-/-} mice.

231 **Compromised synapsis in *Ankrd31*^{-/-} meiocytes**

232 Persistent DSBs and defective synapsis induce oocyte elimination in newborn mice and
233 spermatocyte elimination in pachytene (Barchi et al., 2005; Burgoyne et al., 2009; Di Giacomo et
234 al., 2005). In contrast, deficiencies in maturation of DSBs into COs lead to chromosome
235 alignment defects and an arrest in metaphase I in spermatocytes (Burgoyne et al., 2009).
236 Hence, the complex patterns of elimination of *Ankrd31*^{-/-} meiocytes prompted us to investigate
237 meiotic recombination in *Ankrd31*^{-/-} mice. Both chromosome axis formation and SC formation are
238 used to stage meiotic prophase and provide a reference for kinetic studies of recombination
239 (Table S1). Hence, we tested firstly if chromosome axis, and secondly if SC form with normal
240 kinetics in *Ankrd31*^{-/-} spermatocytes. Axes formed with wild type kinetics (Figure S5A), but SC
241 formation was delayed on autosomes in *Ankrd31*^{-/-} spermatocytes (Figure 3D and 3E). Despite
242 this delay most *Ankrd31*^{-/-} spermatocytes seemed to complete autosomal SC formation in adults
243 (Figure 3D). Due to methodological limitations it is unclear if all spermatocytes succeeded in
244 autosomal synapsis in adults (see STAR Methods for explanation). Autosomal SC formation
245 occurred with high fidelity as we did not observe obvious non-homologous interactions in
246 pachytene stage spermatocytes (n>200) in our *Ankrd31*^{-/-} mutant line. SC formation was
247 compromised in *Ankrd31*^{-/-} oocytes (Figure S5B), which also manifested in higher rates of
248 asynapsis in *Ankrd31*^{-/-} oocytes (54.76%, n=126 oocyte) than wild type oocytes (22.96%, n=135
249 oocyte) at a fetal developmental stage where oocytes are in mid pachytene (Table S5).
250 Together, these observations suggest that whereas ANKRD31 is not required for homology
251 search *per se*, ANKRD31 is required for efficient and timely engagement of homologous
252 chromosomes genome wide in both sexes.

253 Compared to autosomes, we observed a more penetrant defect in SC formation between the
254 PARs of the heterologous sex chromosomes in males. PARs failed to synapse in 99.7% (n>420)
255 and 99% (n=191) of pachytene spermatocytes from adult or juvenile *Ankrd31*^{-/-} mice,
256 respectively. Beyond asynaptic PARs, various abnormal sex chromosome configurations were
257 observed in *Ankrd31*^{-/-} spermatocytes (Figure 3F). This indicates that ANKRD31 plays a more
258 important role in ensuring recombination between the PARs of heterologous sex chromosomes
259 than autosomes.

260 **Delayed formation and resolution of early recombination intermediates in *Ankrd31*^{-/-}** 261 **meiocytes**

262 To examine the possible reasons that underpin deficiencies in synapsis formation in *Ankrd31*^{-/-}
263 meioocytes we monitored kinetics of recombination intermediates (Figure 4 and S5C). Single-
264 stranded DNA ends resulting from DSBs are marked by recombinases RAD51 and DMC1 and
265 the RPA complex (Hunter, 2015). Levels of these markers on DSB repair intermediates change
266 as recombination progresses. Whereas RAD51 and DMC1 tend to mark intermediates in
267 unsynapsed regions (prevalent in leptotene and early zygotene), RPA accumulates to higher
268 levels on intermediates in synapsed regions (prevalent in late zygotene and early pachytene).
269 Focus numbers of all three markers drop as wild type meioocytes progress to late pachytene,
270 indicating completion of DSB repair at this stage. RAD51, DMC1 and RPA foci accumulated with
271 a delay in *Ankrd31*^{-/-} spermatocytes (Figure 4A-E and S5C). Focus numbers remained
272 abnormally low until early zygotene, increasing only after chromosome axes fully formed (late
273 zygotene-like stage). Thereafter, RAD51, DMC1 and RPA foci persisted in abnormally high
274 numbers until late prophase in *Ankrd31*^{-/-} spermatocytes indicative of a DSB repair defect
275 (Figure 4C-E). To assess kinetics of DSB formation and repair we also measured another DSB
276 marker, serine 139-phosphorylated histone H2AX (γ H2AX) (Figure 4F and 4G). γ H2AX
277 accumulation is thought to mainly reflect DSB-dependent ATM activity in early prophase, and
278 DSB- and unsynapsed axes-dependent ATR activity after mid zygotene (Barchi et al., 2008;
279 Bellani et al., 2005; Royo et al., 2013). γ H2AX levels were much lower in *Ankrd31*^{-/-} than wild
280 type spermatocytes in leptotene and early zygotene, which could indicate a delay in DSB
281 formation or reduced ATM activation in response to DSB formation. The analysis of the pre-DSB
282 recombinosome presented below supports the interpretation of a DSB formation delay. In
283 contrast, γ H2AX robustly accumulated on unsynapsed chromatin from late zygotene, consistent
284 with proficient ATR activation on unsynapsed chromatin. This also manifested in the apparently
285 normal accumulation of γ H2AX in the unsynapsed chromatin of sex chromosomes, called the
286 sex body, in pachytene (Figure S5D). We also noted that higher numbers of γ H2AX flares
287 persisted on synapsed chromatin in late pachytene and diplotene spermatocytes in *Ankrd31*^{-/-}
288 mice as compared to wild-type (Figure S5D and S5E). These γ H2AX flares are distinct from the
289 sex body and likely indicate persisting recombination intermediates along synapsed sections of
290 autosomes (Chicheportiche et al., 2007).

291 ANKRD31 had a qualitatively similar effect on the dynamics of DSB repair proteins in oocytes as
292 compared to spermatocytes (Figure S5F-O). Accumulation of RAD51, DMC1 and RPA foci was
293 delayed, and both RPA and γ H2AX signal persisted longer in late prophase in *Ankrd31*^{-/-} oocytes
294 as compared to wild-type.

295 Overall, our data suggest that accumulation of DSB recombination intermediates is delayed in
296 both sexes of *Ankrd31*^{-/-} meiocytes, which delays and/or compromises SC formation.
297 Persistence of recombination foci also indicates defective and/or delayed DSB repair. These
298 defects provide a plausible explanation for elevated apoptosis in pachytene spermatocytes and
299 oocytes of newborn mice. The DSB repair and SC defects appear subtle enough to allow the
300 production of a cohort of oocytes for reproduction, which however cannot maintain high fertility
301 with increasing age. Likewise, the subtlety of the DSB repair defects could explain the survival of
302 a significant cohort of *Ankrd31*^{-/-} spermatocytes beyond prophase.

303 **COs are lost from PAR in *Ankrd31*^{-/-} spermatocytes**

304 A penetrant defect in the PAR synapsis in spermatocytes (see Figure 3F) prompted us to test if
305 a CO formation defect in X and Y PAR could account for the unaligned chromosomes in
306 metaphase arrested *Ankrd31*^{-/-} spermatocytes (Figure 3B). Hence, we examined if *Ankrd31*^{-/-}
307 spermatocytes are defective in the formation of MLH1 marked recombination foci, which are
308 thought to be precursors of most COs in mid and late pachytene (Figure 5A and 5B, Hunter,
309 2015). Whereas MLH1 foci formed on autosomes in normal numbers in most *Ankrd31*^{-/-}
310 spermatocytes, MLH1 foci were always absent from the PARs of sex chromosomes in
311 pachytene cells. We also examined chromosome spreads of metaphase spermatocytes. While
312 in wild-type chiasmata were present between each pair of homologs and between sex
313 chromosomes (n=79 cells), no *Ankrd31*^{-/-} spermatocytes had chiasma between X and Y
314 chromosomes as detected by FISH (n=57 cells, Figure 5C). A small fraction of cells (10.12% of
315 89 cells examined without FISH) also lacked chiasma between an autosome pair. Overall, these
316 observations suggest that MLH1 foci form and mature with high efficiency on autosomes, but
317 never in PARs, in spermatocytes. The loss of PAR-associated COs explains the observed
318 metaphase arrest phenotype of *Ankrd31*^{-/-} spermatocytes. Hence, it is likely that loss of
319 ANKRD31 affects fertility more in males than females because ANKRD31 is required for
320 recombination between male-specific heterologous sex chromosomes.

321 CO formation may fail in PARs due to an absence of DSBs or a repair defect of existing DSBs.
322 To test these possibilities we detected DSB foci (RPA) in combination with a FISH probe that
323 recognizes PAR sequences that border the heterologous parts of X and Y chromosomes (Figure
324 5D). RPA foci were present on at least one of the two PARs in most wild type spermatocytes
325 (91.7%, n=169) but only in a minority of (20.9%, n=67) *Ankrd31*^{-/-} spermatocytes in late zygotene
326 or early pachytene. This observation contrasts globally normal RPA focus numbers in *Ankrd31*^{-/-}
327 spermatocytes in late zygotene and early pachytene (Figure 4E). Thus, ANKRD31 seems to be
328 more critical for DSB formation in PARs than in the rest of the genome.

329 **ANKRD31 is required for the normal spatial distribution of meiotic DSBs**

330 The disproportionately severe reduction of DSB repair focus numbers in PARs as compared to
331 autosomes suggested that the spatial distribution of DSB formation is altered in the absence of
332 ANKRD31. To test this possibility we performed genome-wide mapping of single-stranded DNA
333 ends that result from DSB formation in testes of adult mice. We carried out chromatin-
334 immunoprecipitation of the meiosis-specific recombinase, DMC1, and analyzed the
335 immunoprecipitates by sequencing (ssDNA ChIP-seq, SSDS) (Khil et al., 2012; Smagulova et
336 al., 2011) (Figure 6, S6 and Table S6, S7). SSDS informs on the relative steady state levels and
337 genomic distribution of DMC1-associated single-stranded DNA in early recombination
338 intermediates, which are a function of both DSB formation and the half-life of early
339 recombination intermediates.

340 **An increased usage of default hotspots**

341 DSB hotspot numbers were higher in *Ankrd31*^{-/-} (22215) than wild type control (13507) testes as
342 measured by SSDS. We also observed major differences in the spatial distribution of DSB
343 hotspots between *Ankrd31*^{-/-} testis and wild-type (Figure 6A, 6B and S6A). In *Ankrd31*^{-/-} mice,
344 61% of DSB hotspots overlapped with PRDM9-dependent hotspots of B6 *Ankrd31*^{+/+} (hereafter
345 called B6 hotspots), and 33% overlapped with DSB hotspots of *Prdm9*^{-/-} mice (default hotspots)
346 that mostly associate with active promoters (Brick et al., 2012). A further 6% of the *Ankrd31*^{-/-}
347 hotspots were at previously undescribed sites (hereafter called “new”). In *Ankrd31*^{-/-}, the average
348 strength of hotspots overlapping with B6 hotspots was the highest and “new” hotspots were the
349 weakest (Figure 6C).

350 In addition, a redistribution of the intensity of SSDS signals was observed at hotspots
351 overlapping with B6 hotspots in *Ankrd31*^{-/-} testes. This was illustrated by a lower correlation in
352 hotspot-associated SSDS signals ($r=0.76$, Pearson) between *Ankrd31*^{-/-} and wild-type than
353 between two wild type samples ($r= 0.98$) (Figure 6D and 6E). Consistent with an apparent
354 redistribution of single-stranded DNA signal, not all the B6 hotspots were detected as hotspots
355 in *Ankrd31*^{-/-} testes. Weak B6 hotspots were detected in lower proportions in *Ankrd31*^{-/-} testes
356 than strong B6 hotspots (Figure S6B). Likewise, strong default hotspots of *Prdm9*^{-/-} mice
357 emerged as hotspots in *Ankrd31*^{-/-} mice with higher frequency than weak default hotspots (Figure
358 S6C). The combination of these observations suggested that there was a general shift from the
359 use of PRDM9-dependent B6 hotspots towards default hotspots in *Ankrd31*^{-/-} mice. We conclude
360 that there is a differential hotspot usage, and possibly differential DMC1 loading or ssDNA

361 turnover at hotspots, in *Ankrd31*^{-/-} compared to wild type, which could be influenced by altered
362 timing of recombination initiation.

363 **A strong reduction of DSB activity in the PAR**

364 Given that loss of ANKRD31 altered the distribution of SSDS signal at the hotspot level, we
365 wondered if ANKRD31 may also have differential effects in specific chromosomes and in the
366 PAR. Wild-type to *Ankrd31*^{-/-} SSDS signal ratios were close to one (from 0.96 to 1.11) on each
367 autosome and sex chromosome regions excluding the PAR. In contrast, wild-type to *Ankrd31*^{-/-}
368 ratios were high in the PAR (9.4) and at hotspots within a 150 kb region upstream of X PAR
369 (Figure 6F, 6G and Table S7 see also STAR Methods for mapping reads to PAR). These
370 observations indicate a strong decrease of DSB activity in, and around, the PAR relative to
371 genome average in *Ankrd31*^{-/-} male mice, which supports the hypothesis that ANKRD31 has a
372 particularly critical role in recombination and DSB formation in the PAR.

373 **Histone H3K4me3 distribution on chromatin is similar in wild type and *Ankrd31*^{-/-} testes**

374 Global changes in the timing and the spatial distribution of DSBs in *Ankrd31*^{-/-} mice could be
375 caused by a change in the testicular transcriptome or altered chromatin organisation. However,
376 we detected very few, if any, changes in the testis transcriptome of juvenile (12 days old)
377 *Ankrd31*^{-/-} mice relative to wild-type (Figure S6D and S6E). Testicular PRDM9 protein levels
378 were also similar in *Ankrd31*^{-/-} and wild type testes (Figure S6F). Both PRDM9-dependent and
379 PRDM9-independent hotspots are characterized by enrichment for histone H3K4me3, therefore
380 we performed histone H3K4me3 ChIP-seq on testes of juvenile mice (12 days old) to test if an
381 altered distribution of H3K4me3 could explain the patterns of DSB formation in *Ankrd31*^{-/-} mice
382 (Figure 6A and 6G). To allow better comparison of different samples we normalized H3K4me3
383 signal to a common set of transcription start sites (Davies et al., 2016 and STAR Methods).

384 H3K4me3 signal enrichment appeared highly similar in wild type and *Ankrd31*^{-/-} mice both at B6
385 hotspots and PRDM9-independent hotspots including the broader region of the PAR (Figure 6A,
386 6G, and Table S7). Indeed, histone H3K4me3 enrichment strongly correlated between wild type
387 and *Ankrd31*^{-/-} testes at B6 (r=0.99) and default (r=0.99) hotspot sites (Figure 6H, 6I) indicating
388 that PRDM9-dependent and -independent H3K4me3 depositions are not affected in *Ankrd31*^{-/-}
389 mice. Furthermore, we found no significant correlation between the variation of wild-type to
390 *Ankrd31*^{-/-} SSDS signal ratios and H3K4me3 signal ratios at B6 hotspots (Figure S6G).

391 Together, these observations suggest that ANKRD31 is required for neither PRDM9
392 methyltransferase activity at B6 hotspots nor the accumulation of H3K4me3 at PRDM9-

393 independent hotspots including the PAR. The shift in DSB activity from B6 to default hotspots
394 and the severe reduction of DSB activity in the PAR in *Ankrd31*^{-/-} mice are therefore not due to
395 changes in histone H3K4me3 levels.

396 **Timely assembly of DSB-promoting proteins into chromatin-bound complexes depends** 397 **on ANKRD31**

398 Given its localization on axes, ANKRD31 might modulate the function of pre-DSB
399 recombinosomes, which could provide a feasible explanation for *Ankrd31*^{-/-} phenotypes.
400 Consistent with this hypothesis we found that both MEI4 and REC114 foci appeared with
401 delayed kinetics in *Ankrd31*^{-/-} spermatocytes (Figure 7A-C and S7A-C). Nevertheless, MEI4 and
402 REC114 foci did accumulate by zygotene and persisted until SC formation was complete.
403 Kinetics of MEI4 and REC114 focus formation matched those of early recombination
404 intermediates (RAD51, DMC1 and RPA foci), suggesting that a delay in the formation of MEI4-
405 and REC114-marked pre-DSB recombinosomes contributes to a delay in DSB formation in
406 *Ankrd31*^{-/-} meocytes.

407 **Robust aggregates of DSB-promoting proteins in PARs depend on ANKRD31**

408 Whereas MEI4 and REC114 foci eventually formed in *Ankrd31*^{-/-} meocytes, we did not observe
409 MEI4 and REC114 aggregates in PARs or autosome ends (Figure 7B and S7B). We also
410 noticed an alteration of chromosome axis in PARs in *Ankrd31*^{-/-} meocytes. SYCP3-staining
411 appeared brighter and/or thicker at the PAR-end of sex chromosomes (particularly on Y
412 chromosome) in late zygotene and at the zygotene-to-pachytene transition in wild-type (Figure
413 1F, 7D and S7D, Page et al., 2012). This alteration of PAR axes was also highlighted by a
414 marked enrichment of IHO1, which is both a component of pre DSB-recombinosomes and a
415 recombinosome-independent marker of unsynapsed axes (Stanzione et al., 2016). Thus, while
416 IHO1 was not required for MEI4, REC114 and ANKRD31 aggregates (Figure 2J, S3H and S3I),
417 IHO1 accumulation paralleled formation of MEI4 and REC114 aggregates (Figure 2G and S3C),
418 and also persisted in PARs after synapsis initiation (Figure S7D and S7E).

419 In *Ankrd31*^{-/-} spermatocytes, IHO1 robustly decorated unsynapsed axes, but its PAR-enrichment
420 was strongly reduced; only a weak enrichment of IHO1 could be discerned in a small minority of
421 late zygotene and zygotene-to-pachytene transition cells (15.56%, n=59 cells, n=2 mice).
422 Mirroring this change, the bright/thickened SYCP3 stain was abolished in PARs in *Ankrd31*^{-/-}
423 spermatocytes. Together, these observations suggest that ANKRD31 is required for the
424 establishment of a specialized PAR axis domain that is particularly rich in DSB-promoting
425 proteins.

426 **Discussion**

427 We identify ANKRD31 as a key meiotic protein that regulates spatial and temporal patterns of
428 pre-DSB recombinosome assembly and recombination activity in mice. Our findings are
429 supported by similar observations of Boekhout et al. 2018 and Acquaviva et al. 2019.

430 **ANKRD31 is part of the pre-DSB recombinosome**

431 ANKRD31 colocalizes with highly conserved protein components of pre-DSB recombinosomes
432 (Figure 2, S3) (IHO1, MEI4 and REC114 in mice) (de Massy, 2013; Kumar et al., 2010; Kumar et
433 al., 2018; Stanzione et al., 2016). Although the precise architecture of these complexes is
434 unknown, ANKRD31 association with pre-DSB recombinosomes seems to involve direct
435 interactions with REC114 and IHO1 (Figure 2, S3 and Boekhout et al. 2018). We observed two
436 types of pre-DSB recombinosomes, foci and aggregates. Foci formed genome-wide (Kumar et
437 al., 2010; Stanzione et al., 2016), aggregates formed in PARs and at the distal ends of
438 chromosome 4, 9 and 13, which share homology with PAR as identified by Acquaviva et al.
439 2019, and reconfirmed by us using FISH (unpublished data). We refer to these autosomal sites
440 as PAR-like regions. Pre-DSB recombinosome foci and aggregates have distinct properties.
441 Focus formation requires IHO1 and is enhanced by, but not dependent on, ANKRD31. In
442 contrast, aggregate formation is independent of IHO1 but requires ANKRD31. IHO1 is thought to
443 recruit pre-DSB recombinosomes to axes in most of the genome through its interaction with
444 HORMAD1. Hence, we propose that the mode of pre-DSB recombinosome recruitment to axes
445 is altered in PARs and PAR-like regions. MEI4, REC114 and ANKRD31 are mutually dependent
446 for aggregate formation (Figure S3), and ANKRD31 might be a specificity-factor that recognizes
447 some properties of PARs and PAR-like regions to allow aggregates. Alternatively, ANKRD31
448 might confer specific properties on pre-DSB recombinosome components, enabling them to be
449 enriched in the unique chromatin environment of PAR and PAR-like regions. ANKRD31 also
450 impacts on the organisation of chromosome axes, as a noticeable thickening of axes in PARs
451 depends on ANKRD31 (Figure 7D). The formation of a disproportionately long PAR axis, short
452 DNA loops and extensive axis splitting, which underlies the axis thickening in our study, all
453 depend on ANKRD31 (Acquaviva et al., 2019). Whether ANKRD31 reorganizes PAR chromatin
454 directly or indirectly (via MEI4 or REC114) is unknown.

455 **ANKRD31 may control recombination initiation by regulating pre-DSB recombinosomes**

456 Beyond altered pre-DSB recombinosome formation, our study revealed two clear consequences
457 of ANKRD31 deficiency for recombination initiation. First, a delay in DSB repair focus formation
458 and, second, a change in the localization and relative levels of DSBs along chromosomes as

459 measured by SSDS, with the most noticeable consequences in the PAR. The delay in the
460 accumulation of early recombination intermediates (DMC1, RAD51 and RPA foci) matched the
461 delayed appearance of pre-DSB recombinosome foci in *Ankrd31*^{-/-} spermatocytes. We thus
462 propose that ANKRD31 enhances pre-DSB recombinosome focus assembly, which helps timely
463 initiation of recombination and SC formation between homologs (Figure S7E). A related, but
464 possibly distinct, molecular function of ANKRD31 may permit aggregate formation of DSB-
465 promoting proteins in PARs, thereby ensuring obligate recombination initiation in this region
466 (Figure S7E).

467

468 **Altered DSB localization to PRDM9-independent sites in *Ankrd31*^{-/-} mice.**

469 DSB activity at PRDM9-independent default sites is normally repressed in wild-type by a poorly
470 understood PRDM9-dependent mechanism, which may reflect competition between PRDM9-
471 dependent and independent sites (Brick et al., 2012; Diagouraga et al., 2018). This control
472 seems important, because extensive DSB activity at default sites is thought to cause asynapsis
473 and delay in DSB repair (Brick et al., 2012; Hayashi et al., 2005). One of the striking features of
474 the change in DSB localization in *Ankrd31*^{-/-} mice is the use of default sites, which could
475 contribute to the synapsis defect and delayed DSB repair kinetics in *Ankrd31*^{-/-} mice. It is unlikely
476 that use of default sites is due to changed PRDM9 activity in *Ankrd31*^{-/-} meocytes, since histone
477 H3K4me3 distribution was not significantly changed (Figure 6, S6 and Table S7). We favor the
478 idea that default site usage is a consequence of altered pre-DSB recombinosome regulation.
479 ANKRD31-dependent properties of pre-DSB recombinosome could impact upon competition
480 between PRDM9-dependent and independent sites. Alternatively delayed DSB formation might
481 by itself promote the usage of default sites in *Ankrd31*^{-/-} mice. A potential link between timing of
482 DSB formation and usage of PRDM9-independent sites has been previously observed; PAR-
483 associated DSBs are mostly independent of PRDM9 and form later than autosome-DSBs
484 (Kauppi et al., 2011).

485

486 **DSB control on the PAR**

487 The PAR region is highly enriched for DSBs compared to the genome average (Kauppi et al.,
488 2011; Lange et al., 2016), and we found that this enrichment requires ANKRD31 (Figure 6 and
489 Table S7). Intriguingly, DSBs were lost from the PAR in *Ankrd31*^{-/-} mice independently of histone
490 H3K4me3 enrichment (Figure 6 and Table S7). Enrichment of histone H3K4me3 had previously

491 been linked to high DSB activity in the PAR (Brick et al., 2012) but is not sufficient to ensure high
492 DSB numbers according to our results. We propose that robust DSB formation in the PAR
493 requires an ANKRD31-dependent enrichment of pre-DSB recombinosomes (Figure 7 and S7)
494 and/or ANKRD31-dependent alterations to axis/loop organisation as also suggested by
495 (Acquaviva et al., 2019; Kauppi et al., 2011). Thus, the distribution of pre-DSB recombinosomes
496 on axes seems to have a crucial role in defining genomic sites where DSBs occur.

497 Curiously, for unknown reasons, DSB activity is PRDM9-independent in the PAR in mice (Brick
498 et al., 2012). We speculate that this might have arisen due to the erosion of PRDM9-binding
499 sites during evolution. Such erosion occurs when the template used for the repair of DSBs at
500 hotspots includes polymorphisms that disrupt PRDM9 binding sites (Lesecque et al., 2014;
501 Myers et al., 2010). Since the strength of this effect increases with hotspot strength, high DSB
502 activity in the PAR region is expected to erode PRDM9 sequence motifs faster than in other
503 genomic regions. One way in which species could cope with the loss of PRDM9 binding sites is
504 the concurrent evolution of the PRDM9 protein (Latrille et al., 2017). Another way is to use
505 localized PRDM9-independent DSB initiation, which might require relieving PRDM9-dependent
506 repression of default hotspot sites in this region, without affecting PRDM9 control in the rest of
507 the genome. This could occur by delaying DSB formation in the PAR to a time-window where
508 PRDM9 is inefficient in providing competitive advantage to PRDM9-dependent hotspot sites
509 and/or blocking default hotspots. A non-exclusive mechanism could be the assembly of pre-DSB
510 recombinosome aggregates with unique characteristics, which might permit the establishment of
511 a PAR-specific chromatin environment where PRDM9-independent DSBs are possible. PRDM9-
512 independent formation of DSBs in the PAR is not a universal feature, since there are PRDM9-
513 dependent hotspots in human PARs (Pratto et al., 2014; Sarbajna et al., 2012). It will be
514 interesting to determine whether the high level of accumulation of pre-DSB proteins is an
515 evolutionary conserved feature of PARs, or if it is linked to PRDM9-independence in DSB
516 formation. We predict that high accumulation of pre-DSB recombinosome proteins will promote
517 high DSB density, regardless of the extent of PRDM9 involvement.

518

519 **Conclusions**

520 The behavior of ANKRD31 and the phenotype of *Ankrd31*^{-/-} meicytes show that correct timing
521 and spatial patterning of recombination initiation are dependent on ANKRD31 in mice. Our
522 observations strongly suggest that ANKRD31 performs these functions by modulating the
523 properties, formation kinetics and/or the stability of complexes of essential DSB-promoting

524 proteins localized on chromosome axes. The phenotype of *Ankrd31*^{-/-} mice provides support for
525 the previously untested hypothesis that spatial patterning of recombination initiation is controlled
526 not only by the chromatin environment of potential DSB sites in chromatin loops, but also by the
527 DSB forming machinery that assembles in the topologically distinct compartment of the
528 chromosome axis. This paradigm is best exemplified in the PARs of heterologous sex
529 chromosomes, where obligate recombination seems to depend on ANKRD31-mediated
530 enrichment of DSB promoting proteins and modification of chromatin organisation.

531

532 **ACKNOWLEDGEMENTS**

533 We thank L. Acquaviva, M. Boekhout, M.E. Karasu, M. Jasin and S. Keeney for sharing critical
534 unpublished results; D. Tränkner and M. Munzig for lab support, R. Jessberger for departmental
535 support and sharing ideas and reagents (anti-SYCP3); M. Stevense, G. Pearce and E. Hoffmann
536 for editing and proofreading the manuscript; Transgenic Core of MPI-CBG for CRISPR
537 injections; Experimental Center of MTZ for antibody production and mouse husbandry; BIOTEC
538 Deep Sequencing for RNA-sequencing and bioinformatics support; the MPI-CBG scientific
539 computing facility for last minute bioinformatics support; I MacGregor, A.M Pendes, L.G.
540 Hernandez for testing ANKRD31 antibodies in various mammalian species; K. Daniel for ovarian
541 and testicular RNA preparation; Christine Brun for immune-cytochemistry support; Montpellier
542 Resources Imagerie (MRI) for microscopy, the ENCODE Consortium and the ENCODE
543 production laboratory(s) for mouse tissue transcriptome datasets. The Deutsche
544 Forschungsgemeinschaft (DFG; grants: TO421/3-1/2, TO421/6-1/2, TO421/7-1, TO421/10-1,
545 TO421/5-1, TO421/8-1/2) and HFSP research grant RGP0008/2015 supported F.P., I.D., R.R.,
546 S.V. and A.T. DIGS-BB Program supported A.B. J.C.. C.G. and B.d.M. were funded by grants
547 from the Centre National pour la Recherche Scientifique (CNRS) and the European Research
548 Council (ERC) Executive Agency under the European Community's Seventh Framework
549 Programme (FP7/2007-2013 Grant Agreement no. [322788]). B.d.M. was recipient of the Prize
550 Coups d'Élan for French Research from the Fondation Bettencourt-Schueller. E.T. and M.B.
551 were funded by Telethon Foundation, (Grant GGP12189) and Mission Sustainability Grant,
552 University of Rome Tor Vergata (Grant 141). D.L., P.J. and J.F. were funded by Czech Science
553 Foundation (CSF): grant 13-08078S to J.F. Ministry of Education, Youth and Sports (MEYS): LQ
554 project of the NSP II to J.F. Boehringer Ingelheim GmbH and Austrian Academy of Sciences
555 supported A.S. Medical Research Council grant number MC_UU_00007/6 supported I.R.A.

556

557 **AUTHOR CONTRIBUTIONS**

558 F.P. generated ANKRD31 antibodies and *Ankrd31*^{-/-} mice, characterized *Ankrd31*^{-/-} male
559 phenotype

560 J.C., ChIP-seq bioinformatics

561 E.T., PAR-FISH

562 I.D. assisted characterization of *Ankrd31*^{-/-} male phenotype, performed ANKRD31 colocalization
563 experiments, analyzed PAR-FISH experiments and ANKRD31 localization in *Mei4*^{-/-} mice, and
564 produced H1t antibody

565 R.R., *Ankrd31*^{-/-} female analysis

566 C.G. performed ChIPs, constructed libraries, purified PRDM9 antibody and measured PRDM9
567 protein levels in *Ankrd31*^{-/-}

568 A.B., Y2Hs and immunoprecipitation-WB of ANKRD31

569 S.V.C. validated ANKRD31 antibodies, analysed ANKRD31 localization and apoptosis in testis
570 cryosections

571 A.S., multiple alignments of ANKRD31 sequences

572 M.S., initial Y2Hs and initial IHO1 localization analysis in PAR

573 P.J., D.L. and J.F., chromosome FISH

574 F.J., CRISPR/CAS9 mRNA and gRNA preparation

575 I.R.A., provided fetal ovaries for screening, and copy-edited manuscript

576 M.B. analyzed PAR-FISH experiments, supervised E.T. and revised the manuscript

577 B.d.M. performed ANKRD31 localization experiments in *Mei4*^{-/-} and *Rec114*^{-/-} mice, analyzed
578 data and co-wrote the section on ChIP-seq, introduction and discussion with A.T. and revised
579 the manuscript.

580 A.T. designed experiments, analyzed data, supervised the project and wrote the manuscript with
581 input from other authors.

582

583 **Declaration of Interests**

584 The authors declare no conflict of interest.

585

586 **Figure Legends**

587

588 **Figure 1. ANKRD31 is a meiotic protein that preferentially localizes to unsynapsed axes**

589 **(A-B)** *Ankrd31* transcript levels are shown as transcripts per million (TPM) in **(A)** total RNAs of
590 fetal ovaries at the indicated developmental stages, or **(B)** mixtures of equal quantities of total
591 RNAs of 17 somatic tissues (somatic) or 17 somatic tissues plus adult testes (testis+ somatic).
592 **(C)** DNA staining by DAPI and immunostaining of ANKRD31 and a meiotic chromosome axis
593 marker, SYCP3, in testis sections. Guinea pig antibodies against an N-terminal fragment of
594 ANKRD31 (Gu-ANKRD31 Nt) were used. Seminiferous tubules at stage IX and X are shown;
595 see STAR Methods for staging. Sertoli cells (se), leptotene (le), zygotene (zy), late pachytene
596 (lpa), and diplotene (di) spermatocytes and elongating spermatids (es) are marked. Gu-
597 ANKRD31 Nt antibodies stained some interstitial cells strongly (yellow arrowhead), and Sertoli
598 cell nuclei weakly. We consider these signals non-specific as they were not reproduced by rabbit
599 antibodies against the same ANKRD31 fragment (Rb-ANKRD31 Nt), see Figure S1B. **(D-F)**
600 ANKRD31, SYCP3 and a marker of synapsis, SYCP1 (transverse filament protein), were stained
601 in spread spermatocytes of adult mice. Rb-ANKRD31 Nt antibodies stained ANKRD31. Matched
602 exposed images of ANKRD31 are shown. SYCP3 and SYCP1 were differentially levelled to
603 optimize viewing of overlay images. Insets of late zygotene and zygotene-pachytene
604 spermatocytes are enlarged in **E** and **F**, respectively. X and Y chromosomes and unsynapsed
605 (unsy) and synapsed (sy) regions of an autosome are marked in some images of **D-F**. In **F**,
606 arrowhead and arrow mark a large and a small aggregate of ANKRD31 on the PAR ends of sex
607 chromosomes and the end of an autosome, respectively. Bars, 50 μ m **(C)**, 10 μ m **(D)**, 2.5 μ m **(E,**
608 **F)**. **(G)** ANKRD31 focus counts (Rb-ANKRD31 Nt) in preleptotene (prele), leptotene (le), early
609 zygotene (ezy) and late zygotene (lzy) spermatocytes. Cell numbers (n) and medians (bars) are
610 shown. See also Figure S1, S2 and Table S1.

611 **Figure 2. ANKRD31 colocalizes with DSB-promoting proteins on chromosome axes, and**
612 **forms aggregates in PARs and PAR-like regions**

613 **(A, D, G, J)** Immunostained spermatocyte spreads. MEI4 **(A)** and REC114 **(D)** signals are
614 shifted to the right by three pixels in the enlarged insets. Arrowheads mark co-aggregates of
615 ANKRD31, MEI4 and REC114. **(B, C, E, F)** Quantification of overlap between ANKRD31 and
616 MEI4 **(B, C)** or REC114 **(E, F)** foci in leptotene (le) and early zygotene (ezy) spermatocytes.
617 Overlap was significantly reduced when ANKRD31 images were turned 90 degree (turn, red)
618 relative to MEI4 or REC114 images, Wilcoxon signed rank test, $P < 0.0001$, (****). **(G, left panel)**
619 Arrowheads mark co-aggregates of ANKRD31 and MEI4 in the PAR and at three autosome
620 ends in a spermatocyte at zygotene-to-pachytene transition. **(G, right panel)** Arrow marks PAR
621 in enlarged insets. **(H)** Schematics of ANKRD31 domains and Y2H interactions. Colored boxes
622 mark ankyrin repeats (ANK1 and ANK2), coiled coil domain (CC), and conserved regions 4

623 (CR4) and 5 (CR5). Blue lines and numbers show ANKRD31 residues that interact with the
624 indicated proteins in Y2H. + and ++ mark weak and strong interactions, respectively. (I)
625 ANKRD31 focus counts in preleptotene (prele), leptotene (le), early (ezy) and late (lzy) zygotene
626 spermatocytes. Cell numbers (n) and medians (bars) are shown. Mann–Whitney U test,
627 $0.001 < P < 0.05$ (**) and $P < 0.0001$ (****). (J) *Iho1*^{-/-} spermatocyte with fully formed chromosome
628 axes. Enlarged insets (bottom panel) show a co-aggregate of ANKRD31 and REC114. (A, D, G
629 and J) Bars, 10µm in low resolution images and 5µm in enlargements. See also Figure S3,
630 Table S2 and S3.

631 **Figure 3. SC formation is defective in *Ankrd31*^{-/-} spermatocytes**

632 (A) Targeting of *Ankrd31* exon 6 by CRISPR/Cas9. Sequences of guide RNA (gRNA, PAM
633 (underlined) and exon 6 (capital)/intron6 (lower case) boundaries are shown in wild-type (WT)
634 and mutant lines (mut1 and 2). (B) DAPI staining of DNA in testis sections. Seminiferous tubules
635 with metaphase spermatocytes are shown (stages XII in wild-type, XII-like in *Ankrd31*^{-/-}, see
636 STAR Methods). Sertoli cells (se), zygotene spermatocytes (zy), metaphase cells (m), lagging
637 chromosomes (la) and immature sperm (sp) are marked. The middle panel shows enlarged
638 colour-coded insets. Bars, 50µm (sections) and 5µm (zoom). (C) Quantification of seminiferous
639 tubules that contain metaphase spermatocytes. Number of tubules (n) counted in four
640 experiments, weighted averages of percentages and standard deviation are shown. (D-F) SC
641 (SYCP1) was detected in combination with axis marker (SYCP3) in *Ankrd31*^{+/+} and *Ankrd31*^{-/-}
642 spermatocytes. (D) Indicated categories of SYCP1 staining patterns were quantified in
643 populations of histone H1t-negative spermatocytes that had short axis (equivalent to wild type
644 leptotene), long incomplete axes (early zygotene), or fully formed continuous axes (late
645 zygotene and early pachytene cells). Spermatocytes of adults or 13 day old juvenile mice were
646 examined. Cell numbers (n), standard deviation and the weighted averages of three (adults) or
647 two (juvenile, 13 days old) experiments are shown. (E, F) SYCP3 and SYCP1 stainings in
648 spread spermatocytes. (E) Images represent axis development categories of D. (F) Sex
649 chromosome configurations in wild type and *Ankrd31*^{-/-} spermatocytes. The dominant fraction of
650 *Ankrd31*^{-/-} spermatocytes (76%) has unsynapsed X and Y chromosomes without other apparent
651 abnormalities. Minor fractions have self-synapsed or short Y axes (12.5%), X-autosome fusions
652 (6.6%) or circularized Y (4.8%), n=271 spermatocytes. X (arrows) and Y (arrowhead)
653 chromosomes are marked. (E, F) Bars, 10µm. (C, D) Chi Square test, non-significant (ns) and
654 $P < 0.0001$ (****). See also Figure S4, S5, Table S4, and S5.

655 **Figure 4. Delayed kinetics of recombination foci in *Ankrd31*^{-/-} spermatocytes**

656 (A, B, F) Immunostained spermatocyte spreads. Bars, 10 μ m. Early zygotene cells are shown in
657 A and B. DMC1 (C), RAD51 (D), and RPA (E) focus numbers and total nuclear γ H2AX signal
658 (G) are shown in spermatocytes at stages (C, D, E) leptotene (le), early zygotene (ezy), late
659 zygotene (lzy), early pachytene (epa), mid pachytene (mpa) and late pachytene (lpa), (G) pooled
660 leptotene and early zygotene (le-ezy) or late zygotene (lzy). (C, D, E, G) Cell numbers (n) and
661 medians (bars) are shown. Mann–Whitney U test, non-significant $P>0.05$ (ns), $P>0.01$ (*),
662 $P>0.001$ (**) and $P<0.0001$ (****). See also Figure S5.

663 **Figure 5. PARs fail to engage in *Ankrd31*^{-/-} spermatocytes**

664 (A) Immunostaining in spread mid pachytene spermatocytes, as identified by histone H1t
665 staining (miniaturized H1t stain in, the corner of overlay images). Arrowheads mark MLH1 focus
666 in PAR in enlarged images of X and Y chromosomes. (B) MLH1 focus numbers in mid-to-late
667 pachytene. Cell numbers (n) and medians (bars) are shown. Mann–Whitney U test,
668 $0.01>P>0.001$ (**). (C) FISH stained X and Y chromosomes in metaphase spreads; DAPI
669 stained DNA. (D) PAR FISH combined with SYCP3 and RPA staining in spread spermatocytes.
670 One late zygotene *Ankrd31*^{+/+} and two early pachytene *Ankrd31*^{-/-} are shown. Enlargements of
671 boxes are below respective full nucleus images. White dashed lines mark boundaries between
672 PAR FISH signals and the rest of chromosome X or Y. Arrowheads mark axis-associated RPA
673 foci within or distal to PAR signals. Y chromosome PAR images are enlarged from both *Ankrd31*^{-/-}
674 cells (Y₁ and Y₂). Enlarged image of X chromosome PAR is only shown from one of the cells
675 (X₁), because the X PAR overlays an autosomal axis in the other cell (X₂). Bars, 10 μ m (A, C, D),
676 and 5 μ m (A) or 2.5 μ m (D) in enlargements.

677 **Figure 6. Spatial distribution of DSBs is altered in the absence of ANKRD31**

678 (A, G) DMC1 ChIP SSDS and histone H3K4me3 signal coverage (merged from two
679 experiments) are shown in the indicated mouse lines in a 150kb region of chromosome 1 in A
680 and in the broader PAR region of X chromosome in G. (A) Blue and black arrows mark sites of
681 published C57BL/6J (B6) hotspots and PRDM9-independent default hotspots from Diagouraga
682 et al. 2018 and Brick et al. 2012, respectively. Long and short black arrows represent
683 comparatively strong or weak hotspots in *Prdm9*^{-/-} mice according to DMC1 SSDS coverage. (B)
684 Proportion of *Ankrd31*^{-/-} hotspots that match PRDM9-defined (i.e. B6) or default hotspots, or that
685 do not match either (new). (C) Average plots (top) and heatmaps (bottom) of DMC1 SSDS signal
686 in *Ankrd31*^{-/-} mice at hotspots overlapping with B6, with default (i.e. from *Prdm9*^{-/-}) or with neither
687 of those (new). (D, E) SSDS signal comparison at PRDM9-defined hotspots between *Ankrd31*^{+/+}

688 and *Ankrd31*^{-/-} in **D**, or between *Ankrd31*^{+/+} and wild-type C57BL/6J mice (data from Diagouraga
689 et al. 2018) in **E**.

690 (**F**) *Ankrd31*^{+/+} to ^{-/-} DMC1 SSDS signal ratios are shown for each autosome, the non-PAR
691 regions of X (X^{np}) and Y (Y^{np}), and available sequence of the X PAR. (**G**) Arrows mark PRDM9-
692 independent hotspots in the non-PAR region upstream of PAR. (**H-J**) Comparisons of
693 normalized H3K4me3 signals at PRDM9-defined (B6) (**H**), default (**I**) or new (**J**) hotspots sites
694 between *Ankrd31*^{+/+} and *Ankrd31*^{-/-} mice. (**D, E, H-J**) Dotted red lines represent one to one
695 relationship between x and y. Levels represent the density calculated with the density2d function
696 from R. Pearson's correlation coefficient (r) is indicated with significance (p). See also Figure
697 S6, Table S6 and S7.

698 **Figure 7 ANKRD31 is required for timely MEI4 focus formation and the enrichment of**
699 **DSB-promoting proteins in PARs**

700 (**A, B, D**) Immunofluorescence of indicated proteins and centromere (CENT) in spread
701 spermatocytes in leptotene (**A**) and late zygotene (**B, D**). (**A, B**) MEI4 exposures were matched
702 between genotypes. (**B**) Arrowheads mark MEI4 aggregates. Fractions of late zygotene
703 spermatocytes with MEI4 aggregates are shown in MEI4 images. (**C**) MEI4 focus numbers in
704 preleptotene (prele), leptotene (le), early (ezy) and late (lzy) zygotene spermatocytes. Cell
705 numbers (n) and medians (bars) are shown. Mann-Whitney U test, 0.01>P>0.001 (**), P<0.0001
706 (****). (**D**) Boxed X and Y chromosomes are enlarged in bottom panels, arrows mark PAR,
707 arrowheads mark centromeric ends. Bars, 10µm (**A, B** and **D**) and 5µm (**D** in enlargement). See
708 also Figure S7.

709

710

711 **STAR METHODS**

712 **CONTACT FOR REAGENT AND RESOURCE SHARING**

713 Further information and request for resources and reagents should be directed to the Lead
714 Contact, Attila Toth (attila.toth@mailbox.tu-dresden.de).

715

716 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

717 **Animal experiments, choice of adults or juveniles**

718 Gonads were collected from mice after euthanasia. Most cytological experiments of
719 spermatocytes were carried out on samples collected from adult mice unless indicated
720 otherwise. In particular, we used juvenile mice (13-14 days old) to enrich for cells that are in late
721 zygotene or zygotene-to-pachytene transition. Juvenile mice were also used for transcriptome
722 analysis and histone H3K4me3 ChIPseq, where differences between the cellularities of testes in
723 adult wild type and *Ankrd31*^{-/-} mice would have complicated interpretation of experimental
724 outcomes. The first wave of spermatocytes reaches mid pachytene in 14 days old mice, at which
725 point cellularities of testes in wild type and *Ankrd31*^{-/-} mice begin to differ due to apoptosis of
726 spermatogenic cells in the latter. Transcriptomes and histone H3K4me3 patterns are expected to
727 greatly differ in meiotic and non-meiotic cell populations of testes. Therefore, altered relative
728 proportions of these cell populations differentiate transcriptomes and histone H3K4me3 ChIPseq
729 signal distributions in testes of wild-type and *Ankrd31*^{-/-} mice that are older than 13-14days.
730 Hence, we used 12 days old mice for these experiments.

731 Animals were used and maintained in accordance with the German Animal Welfare legislation
732 ("Tierschutzgesetz"). All procedures pertaining to animal experiments were approved by the
733 Governmental IACUC ("Landesdirektion Sachsen") and overseen by the animal ethics
734 committee of the Technische Universität Dresden. The licence numbers concerned with the
735 present experiments with mice are T 2014-1 and TV A 8/2017.

736 **Generation of *Ankrd31*-knockout mice**

737 *Ankrd31* mutant lines were generated using CRISPR/Cas9 genome editing (Hwang et al., 2013),
738 targeting exon6 of *Ankrd31* gene. A mixture of gRNA: AGTCACCAAAACACTGG (12.5 ng/μl)
739 (designed using the online platform at <http://crispr.mit.edu/>) and Cas9 nuclease mRNA (50 ng/μl)
740 was injected into pronucleus/cytoplasm of fertilized oocytes. The oocytes were subsequently
741 transferred into pseudopregnant recipients. Injections and embryo transfer were performed by
742 Transgenic Core Facility of MPI-CBG (Dresden, Germany). Out of 115 injected embryos, 82
743 were transferred into females and 15 were born. 14 of the 15 born pups had alterations in the
744 targeted genomic locus. Two mice that were heterozygote for predicted frame-shift causing
745 alleles (mut1 and mut2, Figure 3A) were bred with C57BL/6JCrI wild type mice to establish
746 mouse lines. All experiments reported in the manuscript are based on samples from mice that
747 were derivative of founder lines after at least three backcrosses.

748

749 **METHOD DETAILS**

750 **CRISPR/Cas9 procedures**

751 gRNA production

752 The guide RNA (gRNA) expression vector DR274 (Addgene #42250) was used to construct the
753 gRNA (Hwang et al., 2013). Primers encoding gRNA sequence were annealed to form double-
754 strand DNA with overhangs for ligation into DR274 vector that was linearized with BsaI
755 restriction enzyme (NEB). PCR product was amplified from the resulting plasmid with
756 DR274_F/DR274_R primers and used as a template for *in vitro* transcription with MEGAscript™
757 T7 Transcription Kit (Ambion).

758 CAS9 mRNA preparation

759 To prepare Cas9 mRNA, we first used the restriction enzyme PmeI to linearize the plasmid
760 MLM3613 (Addgene #42251) (Hwang et al., 2013) that harbors a codon optimized Cas9 coding
761 sequence and a T7 promoter for Cas9 mRNA *in vitro* synthesis. We then used the linearized
762 MLM3613 as template to synthesize the 5' capped and 3' polyA-tailed Cas9 mRNA using the
763 mMESAGE mMACHINE® T7 Ultra Kit (ThermoFisher, cat no: AM1345) according to the
764 manufacturer's instructions.

765 **Genotyping *Ankrd31*-knockout mice**

766 Tail biopsies were used to generate genomic DNA by overnight protease K digestion at 55°C in
767 lysis buffer (200mM NaCl, 100mM Tris-HCl pH 8, 5mM EDTA, 0.1% SDS). Following heat
768 inactivation for 10 min at 95°C. These genomic preparations were used for PCR. F0 mice were
769 genotyped by PCR amplification followed by PAGE electrophoresis and DNA sequencing. Mice
770 in subsequent crosses were genotyped by PCR amplification followed by agarose gel
771 electrophoresis. Combination of four primers, AS-79WT-FW2, Ank_ex5gen_LngR,
772 Ank_ex5gen_LngFW, KO79-RV, were used to genotype the *Ankrd31^{mut1}* allele. PCR product
773 sizes were 623bp and 262bp for wild type, and 624bp and 400bp for *Ankrd31^{mut1}* allele. Two
774 primers, Ank_ex5_gen_shFW and Ank_ex5_gen_shR, were used to genotype *Ankrd31^{mut2}*
775 allele. PCR product sizes were 281bp for wild type, and 248bp for *Ankrd31^{mut2}* allele.

776 **Generation of antibodies against fragments of ANKRD31, Histone H1t and REC114**

777 Antibodies were raised against two ANKRD31 fragments (N-terminal fragment, 144 amino acids
778 between Thr182 and Met325 residues, C-terminal fragment, 153 amino acids between Pro1470
779 and Arg1622 residues), full length Histone H1t, and two REC114 fragments (N-terminal, 130
780 amino acids between Met1 and Glu130 residues, C-terminal fragment, 129 amino acids between
781 Arg131 and Asn259 residues). Coding sequences corresponding to these peptides were cloned
782 into pDEST17 bacterial expression vector. Recombinant 6xHis-tagged proteins were expressed
783 in *E. coli* strain, BL21 tRNA (ANKRD31) or BL21(DE3)pLysS (Histone H1t and REC114) , and

784 subsequently purified on Ni-Sepharose beads (Cat. no. 17-5318-01, Amersham, GE
785 Healthcare). Purified proteins were used for immunization of rabbits and guinea pigs. ANKRD31
786 fragments coupled to NHS-Activated Sepharose 4 Fast Flow beads (Cat. no. 17-0906-01,
787 Amersham, GE Healthcare) were used to affinity purify polyclonal antibodies following standard
788 procedures.

789 **ANKRD31 immunoprecipitation and western blotting**

790 For preparation of protein extracts from wild type and *Ankrd31*-deficient testes, testes of 12 days
791 old juvenile mice were detunicated and homogenized in a lysis buffer (50 mM Tris-HCl pH 7.5,
792 150 mM NaCl, 0.5% Triton X-100, 1 mM MgCl₂). Lysis buffer was supplemented with protease
793 inhibitors and phosphatase inhibitors: 1 mM Phenylmethylsulfonyl Fluoride (PMSF); complete™
794 EDTA-free Protease Inhibitor Cocktail tablets (Roche, 11873580001); 0.5 mM Sodium
795 orthovanadate; Phosphatase inhibitor cocktail 1 (Sigma, P2850) and Phosphatase inhibitor
796 cocktail 2 (Sigma, P5726) were used at concentrations recommended by the manufacturers.
797 Testis homogenates were lysed for 60 min at 4°C in the presence of benzonase (Merck
798 Millipore) to digest DNA during lysis. Lysates were spun at 1000 g for 10 min. Supernatants
799 were diluted two times with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, mixed with 1 µg of rabbit
800 anti-ANKRD31 C-terminal antibody and incubated for 2.5 h at 4°C. 1.5 mg of Dynabeads™
801 Protein A (Invitrogen) were added to the lysate-antibody mix and incubated for 4 h at 4°C. Beads
802 were washed twice with washing buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.25% Triton X-
803 100). Immunoprecipitated material was eluted from the beads by incubating the beads in 100 µl
804 Laemmli sample buffer for 10 min at 70°C. The proteins from resulting elutions and input
805 samples were separated on 4-15% TBX-acrylamide gradient gel (Bio-Rad) and blotted onto
806 PVDF membrane (Sigma, P2938). Membranes were blocked for 1 h at room temperature using
807 blocking solution (5% skimmed milk, 0.05% Tween 20, in TBS pH 7.6) and incubated overnight
808 at 4°C with guinea pig anti-ANKRD31 N-terminal (1:1000) and mouse anti-GAPDH (1:1000)
809 primary antibodies diluted in 0.05% Tween 20 in TBS pH 7.6 (TBS-T). Afterwards, horseradish
810 peroxidase (HRP)-conjugated secondary antibodies (diluted in 2.5% skimmed milk in TBS-T)
811 were applied for 1 h at room temperature. Detection of secondary antibodies was performed with
812 Immobilon Western Chemiluminescent HRP Substrate (Millipore).

813 **Cytoplasmic and nuclear fractionation for PRDM9 detection**

814 Juvenile mouse testes were homogenized in hypotonic buffer (10 mM HEPES, pH 8.0, 320 mM
815 sucrose, 1 mM PMSF, 1x Complete protease inhibitor cocktail EDTA-free (Roche, Cat. Number
816 11873580001)) and phosphatase inhibitor 1x (Thermo Scientific, Halt Phosphatase Inhibitor
817 Cocktail, Cat Number 78420) in a glass douncer. Testis cell suspensions were centrifuged at

818 1000g at 4°C for 10 min. Supernatants were collected and used as cytoplasmic fractions. Pellets
819 were resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-
820 40, 0.5% Na-deoxycholate, 0.1% SDS, 1x Complete protease inhibitor EDTA-free (Roche)) and
821 sonicated (4 cycles of 15s ON, 15s OFF, high power) on a Bioruptor Next-Gen sonicator
822 (Diagenode). Suspensions were centrifuged at 16000 g, 4°C for 10min Then, supernatants were
823 collected and used as nuclear fractions. Cytoplasmic and nuclear fractions (40 µg) were
824 separated on a 4-15% TBX-acrylamide gradient gel (Bio-Rad) and blotted onto nitrocellulose
825 membranes. The membrane was blocked for 1 h at room temperature (1xTBS-T / 5% Milk) and
826 cut according to expected sizes. The upper part was incubated over night at 4°C with affinity
827 purified rabbit anti-PRDM9 (1:1000) (Grey et al., 2017) and guinea pig anti SYCP3 (1:3000)
828 raised against mouse SYCP3 residues 24-44. Secondary antibodies were goat anti-rabbit IgG-
829 HRP (1:5000) (1858415, Pierce) and goat anti-guinea pig IgG-HRP (1:5000) (706-035-148,
830 Jackson Immuno Research). Blots were revealed with Super Signal West Pico
831 Chemiluminescent Substrate, 34080, Thermo Scientific).

832 **Yeast two-hybrid (Y2H) assay**

833 Yeast two-hybrid experiments were performed as described previously with minor modifications
834 (Stanzione et al., 2016). Pairwise interactions were tested in the Y2HGold Yeast strain (Cat. no.
835 630498, Clontech). To transform Y2HGold with bait and prey vectors, yeasts were grown in
836 2xYPDA medium overnight at 30°C, 200 r.p.m. shaking. Afterwards, yeast cells were diluted to
837 0.4 optical density (measured at 600 nm) and incubated in 2xYPDA for 5 h at 30°C, 200 r.p.m.
838 shaking. Cells were harvested, washed with water and resuspended in 2 ml of 100 mM lithium
839 acetate (LiAc). 50 µl of this cell suspension was used for each transformation. Transformation
840 mix included 1 µg of each vector (bait and prey), 60 µl of polyethylene glycol 50% (w/v in water),
841 9 µl of 1.0M LiAc, 12.5 µl of boiled single-strand DNA from salmon sperm (AM9680, Ambion),
842 and water up to 90 µl in total. The transformation mix was incubated at 30°C for 30 min, and
843 then at 42°C for 30 min for the heat shock. The transformation mix was removed following
844 centrifugation at 1000 g for 10 min, and then cells were resuspended in water, and plated first on
845 -Leu -Trp plates to allow selective growth of transformants. After 2 days of growth, transformants
846 were plated both on -Leu -Trp and -Leu -Trp -Ade -His plates for 2-7 days to test for interactions.
847 We followed the manufacturer's instructions for media and plate preparation. The full length
848 ANKRD31 protein activated the Y2H reporter system even in the absence of potential binding
849 partner proteins from mice, which prevented the use of full length ANKRD31 in Y2H. To
850 overcome this limitation, an array of fragments that cover the full length of ANKRD31 sequence
851 were used in Y2H (Table S2). Self-activation tests showed that interactions could be tested

852 along the entire length of ANKRD31 by using a combination of ANKRD31 prey and bait
853 constructs.

854 **Immunofluorescence microscopy**

855 Preparation of spermatocyte spreads

856 Preparation and immunostaining of nuclear surface spreads of spermatocytes was carried out
857 according to earlier described protocols with minor modifications (Peters et al., 1997; Stanzione
858 et al., 2016). Briefly, testis cell suspensions were prepared in PBS pH 7.4, then mixed with
859 hypotonic extraction buffer in 1:1 ratio and incubated for 8 min at room temperature. After
860 diluting the cell suspension five times in PBS pH 7.4, cell suspensions were centrifuged for 5 min
861 at 1000 g, and cells were resuspended in the 1:2 mixture of PBS and 100mM sucrose solution.
862 Cell suspensions were added to seven times higher volume droplets of filtered (0.2 µm) 1%
863 paraformaldehyde (PFA), 0.15% Triton X-100, 1mM sodium borate pH 9.2 solution on diagnostic
864 slides, and incubated for 60 min at room temperature in wet chambers. Nuclei were then dried
865 for at least 1 h under fume-hood. Finally, the slides were washed in 0.4% Photo-Flo 200 (Kodak)
866 and dried at room temperature.

867 Preparation of oocyte spreads

868 To prepare nuclear surface spread oocytes, two ovaries from each mouse were incubated in 20
869 µl hypotonic extraction buffer for 15 min (Hypotonic Extraction Buffer/HEB: 30 mM Tris-HCl, 17
870 mM Trisodium citrate dihydrate, 5 mM EDTA ,100 mM sucrose, 0.5 mM DTT, 0.5 mM PMSF,
871 1xProtease Inhibitor Cocktail). After incubation, HEB solution was removed and 16µl of 100 mM
872 sucrose in 5mM sodium borate buffer pH 8.5 was added. Ovaries were punctured by two
873 needles to release oocytes. Big pieces of tissue were removed. 9µl of 65 mM sucrose in 5 mM
874 sodium borate buffer pH 8.5 was added to the cell suspension and incubated for 3 min. After
875 mixing, 1.5µl of the cell suspension was added in a well containing 20µl of fixative (1%
876 paraformaldehyde/50mM borate buffer pH:9.2/0.15% Triton-X100) on a glass slide. Cells were
877 fixed for 45 min in humid chambers, then slides were air dried. Upon completion of drying slides
878 were washed with 0.4% Photo-Flo 200 solution (Kodak, MFR # 1464510) for 5 min and
879 afterwards they were rinsed with distilled water.

880 Immunofluorescence on gonad sections

881 To detect ANKRD31, testes were sectioned before fixation. Collected testes were immediately
882 frozen in OCT (Sakura Finetek Europe) and sectioned (8 µm thick) on a cryostat. Slices of testes
883 were allowed to dry on glass slides followed by fixation for 30 minutes at room temperature in
884 4% paraformaldehyde in phosphate buffer pH 7.4 followed by permeabilization in PBS/0.2%

885 Triton-X100 for 10min. Slides were washed in PBS 3 times, and blocked in blocking buffer (5%
886 BSA in PBS, 0.05% tween-20, 0.05% Triton X-100) before staining with anti-ANKRD31 and anti-
887 SYCP3 antibodies, followed by DAPI staining. Anti-SYCP3 and DAPI staining served to facilitate
888 exact staging of prophase in spermatogonial cells.

889 To detect apoptosis in testis or ovary sections, we sectioned testes both before and after fixation
890 and ovaries only after fixation. To prepare sections of gonads after fixation, testes from adults
891 and ovaries from newborn mice were fixed in 3.6% formaldehyde in PBS pH 7.4, 0.1% Triton X-
892 100 at room temperature for 40 min (testes) or 20min (ovaries). After fixation testes/ovaries were
893 washed 3 times in PBS pH 7.4 and placed in 30% sucrose overnight at 4°C. Fixed testes/ovaries
894 were frozen on dry ice in OCT (Sakura Finetek Europe). 8 µm thick sections (testes) and 5 µm
895 thick sections (ovaries) were cut and dried onto slides. For ovary sections, an additional step of
896 permeabilization was performed by incubating the slides for 10 min in methanol and 1 min in
897 acetone at -20°C. The sections were washed in PBS pH 7.4 and immediately used for
898 immunofluorescence staining. Anti-cleaved PARP staining (apoptosis marker) and histone H1t
899 (post-mid pachytene stage marker) were detected by immunofluorescence in testes sections.
900 DNA was counterstained by DAPI to facilitate staging of seminiferous tubules. Anti-cleaved
901 PARP and GCNA1 (oocyte marker) (Enders and May, 1994) were detected on oocyte sections.
902 The numbers of cleaved PARP-positive and -negative oocytes were counted on every seventh
903 section to determine the proportion of apoptotic oocytes.

904 To assess oocyte numbers in adult mice DDX4 was detected in paraffin-embedded sections of
905 ovaries in young adults (6-7weeks old). Ovaries were dissected and fixed in 4%
906 paraformaldehyde in 100 mM Sodium Phosphate buffer pH 7.4 overnight at 4°C. Afterwards,
907 ovaries were washed 3 times in PBS pH 7.4, once with 70% ethanol and embedded in paraffin
908 for sectioning at 5 µm thickness. Deparaffinization and rehydration of the sections was
909 performed as follows: 2 x 5 min in xylene, 2 x 5 min in 100% ethanol, 5 min each in 95%, 85%,
910 70%, 50% ethanol, 2 x 5 min in water. Sections were subjected to heat-mediated antigen
911 retrieval in 10 mM Sodium citrate, 0.05% Tween 20, pH 6.0 for 20min on boiling water bath.
912 Sections were permeabilized in PBS with 0.2% Triton X-100 for 45 min at room temperature and
913 processed for immunofluorescence staining immediately. DDX4-positive oocytes were counted
914 on every seventh section of both ovaries in each female mouse.

915 Staining procedures

916 Previously described blocking and immunostaining procedures (Stanzione et al., 2016) were
917 optimised for immunostaining with each combination of antibodies, details are available upon
918 request.

919 Quantification of immunofluorescence signal levels and focus counts

920 Background corrected γ H2AX signal was quantified in whole nucleus with a similar strategy as
921 described earlier (Daniel et al., 2011). DSB repair foci were counted “manually” on matched
922 exposure images of wild type and *Ankrd31*^{-/-} nuclear spreads of meiocytes. Focus numbers of
923 pre-DSB recombinosome proteins (MEI4, REC114 and ANKRD31) and the co-localization of foci
924 were quantified by Cell Profiler software as described previously for the analysis of MEI4,
925 REC114 and IHO1 foci (Stanzione et al., 2016).

926 **Immunofluorescence staining combined with pseudoautosomal region (PAR)**
927 **Fluorescence *in situ* hybridization (FISH)**

928 After 10 min wash with Washing Buffer 1 (WB1, 0.4% Photo-Flo 200, 0.01% Triton X-100 in
929 water), surface spreads were incubated overnight at room temperature with the primary antibody
930 diluted in antibody dilution buffer (ADB) (10% goat serum, 3% bovine serum albumin [BSA],
931 0.05% Triton X-100 in phosphate-buffered saline [PBS]).

932 Then, slides were washed in 10 min WB1 and 10 min Washing Buffer 2 (WB2 0.4% Kodak
933 Photo-Flo 200 in water), and incubated with the secondary antibody for 60 min in a pre-warmed
934 humidified chamber at 37°C in the dark. Following further 10 min WB1, 10 min WB2 and 1 min
935 PBS washes, slides were incubated in Hoechst 33258/PBS solution for at least 20 min in a room
936 temperature humidified chamber. Air-drying slides for 10 min at room temperature in the dark,
937 coverslips were mounted using ProLong® Gold Antifade Mountant without DAPI (Molecular
938 Probes- Life technologies cat. num. P36934). Images were captured using Leica CTR6000
939 Digital Inverted Microscope connected to a charge-coupled device camera and analyzed using
940 the Leica software LAS-AF, for fluorescent microscopy.

941 Preparation of clone DNA and nick translation

942 BAC clone for PAR region (RP2450014) were grown in standard Luria Bertani (L.B) medium at
943 32°C for a minimum of 16 h. DNA purification was obtained with Plasmid Maxi-Prep Kit (Qiagen).
944 Determination of DNA concentration was made by both UV spectrophotometry at 260 nm and
945 quantitative analysis on agarose gel. 1 μ g of extracted BAC DNA was marked with fluorescent
946 green-dUTP (Enzo Life Sciences, ref 02N32-050) using Nick Translation Kit (Abbott Molecular)
947 according to the manufacturer’s instructions. For FISH experiment, marked probe was
948 precipitated in EtOH 100%, 3 mM Sodium Acetate pH 5.2 and 0.1 μ g/ μ l mouse Cot-1 DNA
949 (Invitrogen) at -80°C overnight. Resulting pellet was suspended in hybridization buffer (Enzo Life
950 Sciences) according to the manufacturer’s instructions and denatured at 73°C for 5 min in a
951 water bath.

952 PAR Fluorescence *in situ* hybridization (FISH)

953 Following immunofluorescence, spread slides were washed with fresh PBS at RT for 5 min,
954 rinsed briefly in dH₂O and dehydrated passing through an ethanol series and air-dried. After
955 aging (65°C for 1 h), slides were denatured for 7 min in 70% formamide/2×SSC solution at 72°C
956 and immediately dehydrated, passing it through –20°C cooled ethanol series and air-dried. FISH
957 probe was applied to the slides for denaturation and hybridization steps in a humid chamber
958 (75°C for 10 min and 37°C for at least 16 h, respectively). Following two washes with stringent
959 wash buffer (4×SSC/0.2%Tween-20) at 55°C, slides were dehydrated through an ethanol series
960 and air-dried. Nuclei were stained with Hoechst 33258/PBS solution for 20 min at RT and
961 coverslips were mounted using Antifade Mountant without DAPI. Images were captured using
962 Leica CTR6000 digital inverted microscope and analyzed using Leica software LAS-AF.

963 **Immunofluorescence staining combined with chromosome FISH**

964 Testes from 14 days old mice (C57BL/6J) were dissected and spread nuclei of spermatocytes
965 were prepared as described earlier (Peters et al., 1997) with minor modifications. Briefly, a
966 single-cell suspension of spermatogenic cells in 0.1 mM sucrose with protease inhibitors
967 (Roche) was dropped on 1% paraformaldehyde-treated slides and allowed to settle for 3 h in a
968 humidified box at 4°C. After brief washing with water and PBS, the spread nuclei were blocked
969 with 5% goat serum in PBS (vol/vol), the cells were immunolabeled in a humid chamber at 4°C
970 for 12 h with specific antibodies: anti-ANKRD31 N-terminal domain (diluted 1:400, rabbit
971 antibody), anti-SYCP3 (1:50, mouse monoclonal antibody, Santa Cruz #74569) and anti-CENT
972 (1:200, human – purified antibodies from human anti-centromere positive serum, Antibodies
973 Incorporated #15-235). Secondary antibodies were used at 1:500 dilutions and incubated at 4°C
974 for 90 min; goat anti-Rabbit IgG-AlexaFluor488 (MolecularProbes, A-11034), goat anti-Mouse
975 IgG-AlexaFluor594 (MolecularProbes, A-11032), and goat anti-Human IgG-AlexaFluor647
976 (MolecularProbes, A-21445).

977 After the immunofluorescence staining, the slides were used for Fluorescence *in situ*
978 hybridization (FISH) as described (Kauppi et al., 2011) with DNA FISH probes for mouse
979 chromosomes 4, 9 and 13. Briefly, the slides were dehydrated, denatured and hybridized in a
980 humid chamber at 37°C for 60 hours with one of three mouse chromosome painting probes with
981 an orange emitting fluorochrome (Meta Systems probes): 1. XMP 4 orange (# D-1404-050-OR);
982 2. XMP 9 orange (# D-1409-050-OR); 3. XMP 13 orange (# D-1413-050-OR). After FISH, the
983 slides were washed in stringency wash solution, drained and mounted in Vectashield-DAPI+
984 mounting medium.

985 The images were examined by Nikon Eclipse 400 microscope with a Plan Fluor objective 60x
986 (MRH00601, Nikon) with a setup of five filter blocks: UV (Ex330-380/ Em400-420), FITC (Ex465-
987 495/ Em505-520), ET Orange (Ex530-560/ Em580-600), Sp107/TR (Ex590-615/ Em615-645),
988 CY5 (Ex620-660/ Em660-700). The images were captured using a DS-QiMc monochrome CCD
989 camera (Nikon) and the NIS-Elements program (Nikon). The acquired images were processed
990 with NIS-Elements program and saved in TIF for each channel.

991 **Diakinesis/Metaphase I chromosome spreading**

992 Chromosome spreads of diakinesis/metaphase I stage spermatocytes were prepared as
993 described in (Holloway et al., 2010). Briefly, testes were decapsulated and tubules were
994 disrupted in hypotonic buffer (1% trisodium citrate in water). Large clumps were removed and
995 the cell suspension was incubated in hypotonic buffer for 20min at room temperature. Cell
996 suspension was centrifuged at 200g for 10min and supernatant was removed. Afterwards, cells
997 were fixed in a methanol/acetic acid/chloroform (3:1:0.05 ratio) fixative, centrifuged and
998 resuspended in ice-cold methanol/acetic acid solution (3:1 ratio). Fixed cells were dropped onto
999 slides, dried quickly (in humid conditions), and stained with Hoechst 33342.

1000 **XY chromosome painting in metaphase spreads**

1001 For sex chromosome painting, probes specific for X and Y chromosomes were used (XMP X
1002 Green, XMP Y Orange, MetaSystems). FISH protocol was performed according to
1003 manufacturer's instructions.

1004 **Staging of meiotic prophase**

1005 The first meiotic prophase can be subdivided into stages by a combination of three markers,
1006 SYCP3 (chromosome axis marker), SYCP1 (SC marker) and Histone H1t (post-mid pachytene
1007 marker in spermatocytes) (Table S1, for details see also (Stanzione et al., 2016). The stages
1008 that can be distinguished by these markers are listed briefly below, for a more detailed
1009 description see (Stanzione et al., 2016). Preleptotene stage corresponds to the premeiotic DNA
1010 replication in the cell cycle of germ cells. In a previous study (Stanzione et al., 2016), we used
1011 EdU labeling in combination with SYCP3 stain to define axis morphology that characterizes
1012 preleptotene spermatocytes. Based on this earlier study we define preleptotene as a stage
1013 where hazy/punctate staining pattern of SYCP3 is observed throughout the nucleus. The next
1014 stage, leptotene, is characterized by short stretches of axes and no SC. This is a stage where
1015 recombination is initiated in wild-type. The next stage is early zygotene, which is characterized
1016 by long, yet still fragmented, axis stretches. SC is also detected in this stage in SC proficient
1017 genotypes. During late zygotene, axes of all chromosomes are fully formed but SCs are

1018 incomplete. Cells enter pachytene when SC formation is completed in wild-type. All
1019 chromosomes are fully synapsed in pachytene oocytes. In contrast, only autosomes synapse
1020 fully and heterologous sex chromosomes synapse only in their PARs in spermatocytes. We also
1021 define a zygotene-to-pachytene transition stage for spermatocytes, where up to one autosome
1022 pair has not finished synapsis and/or sex chromosomes were still unsynapsed or about to
1023 synapse in wild-type. Sex chromosome axes, in particular X chromosome axes, are long and
1024 stretched out in this stage. Histone H1t staining is used to sub-stage pachytene. Histone H1t is
1025 absent or weak in early pachytene. Histone H1t levels are intermediate and high in mid and late
1026 pachytene, respectively. Pachytene is followed by the diplotene stage, during which axes
1027 desynapse and the SC becomes fragmented. Histone H1t levels are high in this stage in
1028 spermatocytes. In oocytes the same stages exist but histone H1t cannot be used as a staging
1029 marker. Instead the developmental time of fetuses can be used to aid staging. Most oocytes are
1030 in zygotene and mid-pachytene in fetuses 16 and 18 days postcoitum. Most oocytes are in
1031 pachytene/diplotene in newborn mice.

1032 Staging of prophase is not straightforward in mutants that have SC defects. We used axis
1033 morphology to unequivocally define boundaries between leptotene, early zygotene and late
1034 zygotene (see Table S1). However, by itself, axis morphology cannot be used to unambiguously
1035 distinguish stages after late zygotene. This is because fully formed axis characterizes stages
1036 between late zygotene and early diplotene, and SC is not a reliable marker in cells where SC
1037 formation is incomplete. We used histone H1t levels to aid staging in spermatocytes with fully
1038 formed axes. This allowed us to unambiguously identify pre late pachytene stages by the lack of
1039 histone H1t staining. Given the delayed/defective SC formation in *Ankrd31*^{-/-} mice, *Ankrd31*^{-/-}
1040 spermatocytes that have fully formed axis, incomplete SC, and low histone H1t levels could
1041 correspond to late zygotene or an abnormal early pachytene. For the purpose of our study we
1042 categorised these cells as late zygotene. We note that this ambiguity does not influence our
1043 conclusions about kinetics of pre-DSB recombinosome formation, recombination initiation or
1044 DSB repair. This is because our conclusions rely on either the unambiguous differentiation
1045 between leptotene, early zygotene and late zygotene stages based on axis morphology or the
1046 unambiguous differentiation between early pachytene and late pachytene based on histone H1t
1047 staining. Given the PAR synapsis defect in *Ankrd31*^{-/-} spermatocytes we defined zygotene-to-
1048 pachytene transition as a stage where up to one autosome pair is not fully synapsed in *Ankrd31*^{-/-}
1049 spermatocytes. However, we note that some of these cells might be early pachytene cells with
1050 defective synapsis. Pachytene was defined as a stage where all autosomes are fully synapsed
1051 but sex chromosomes may or may not be synapsed in *Ankrd31*^{-/-} spermatocytes.

1052 **Staging of mouse seminiferous tubule cross sections**

1053 Spermatogenic cells that are located in the same section of a seminiferous tubule initiate and
1054 execute meiosis on a coordinated manner in mice. Meiotic entry occurs in spermatogenic cell
1055 layers at the perimeter of seminiferous tubules. Upon progression in meiosis, spermatogenic
1056 cells move towards the lumen of tubules. Concurrently, mitotic proliferation generates a new
1057 layer of spermatogenic cells for a new wave of meiosis at the perimeter of each seminiferous
1058 tubule. The combination of repeated meiosis entry and spermatogenic cells migration to the
1059 lumen generates the so called epithelial cycle of seminiferous tubules. In mice, the seminiferous
1060 epithelial cycle has 12 well-defined stages (I-XII) which are characterized by distinct associations
1061 of premeiotic, meiotic and post meiotic spermatogenic cell layers across crosssections of
1062 seminiferous tubules (Ahmed and de Rooij, 2009). As spermatogenesis progresses, each
1063 portion of seminiferous tubules transits from stage I to XII, and then to stage I to start a new
1064 cycle. Nuclear morphology and chromatin condensation patterns differ in distinct stages of the
1065 spermatogenic process, hence detection of chromatin by DAPI was used to identify
1066 spermatogenic cell associations that define distinct stages of the seminiferous epithelial cycle. In
1067 some cases we also immunostained SYCP3 (axis) or histone-H1t (marker of spermatocytes
1068 after mid pachytene) to aid staging of the epithelial cycle. Staging without molecular markers of
1069 chromosome axis and SC suggested that stage X of epithelial cycle is characterized by the
1070 combination of elongating spermatids in the lumen, a mixture of late pachytene and diplotene
1071 cells in an intermediate cell layer, and spermatocytes that transit from leptotene to zygotene in
1072 the basal layer of seminiferous tubules (Ahmed and de Rooij, 2009). Immunostaining of axis,
1073 unsynapsed axis and/or SC markers (Figure 1C, S4E and our unpublished observations)
1074 suggested that elongating spermatids are present mostly in combination with diplotene and
1075 zygotene cells in our strain background. Hence we labeled basal and intermediate cell layers as
1076 zygotene and diplotene in Figure 1C and S4E.

1077 Stage XII seminiferous tubules have late zygotene spermatocytes in the basal cell layer,
1078 spermatocytes undergoing meiotic divisions in the intermediate cell layer, and advanced
1079 elongated spermatids in the lumen. In contrast, the basal layer of stage I-IV tubules have early
1080 pachytene cells and increasing numbers of intermediate spermatogonia as tubules progress
1081 from stage I to IV. Stage I-IV tubules contain early round spermatids in the intermediate layer
1082 and advanced elongated spermatids in the luminal layer. Chromatin and SYCP3 staining appear
1083 similar in late zygotene and early pachytene cells. Hence it is difficult to distinguish stage XII
1084 from stage I based on spermatocytes in the basal cell layer. Spermatogonia numbers and
1085 morphology are also similar at these stages. The main distinguishing feature of stage XII is the

1086 presence of metaphase I to anaphase II stage spermatocytes. However, *Ankrd31*^{-/-}
1087 spermatocytes fail to progress beyond metaphase I efficiently, hence intermediate and luminal
1088 cell layers cannot be used to unambiguously distinguish seminiferous tubules in stage XII and
1089 stage I in *Ankrd31*^{-/-}. We referred to seminiferous tubules that contained metaphase I
1090 spermatocytes as “stage XII-like” in *Ankrd31*^{-/-} to indicate this ambiguity.

1091 **Quantification of unaligned chromosomes in mouse seminiferous tubule cross sections**

1092 To quantify the fraction of spermatocytes that had unaligned chromosomes we selected
1093 metaphase I spermatocytes with metaphase plates that appeared perpendicular to the plane of
1094 sectioning. We selected only cells that were clearly separable from other metaphase plates or
1095 nuclei. All the focal plains of the selected metaphase cells were inspected under microscope to
1096 ensure detection of unaligned chromosomes. We note that sectioning may have trimmed
1097 metaphase plates in some cells thereby removing unaligned chromosomes. Hence, unaligned
1098 chromosome counts from sections are underestimates of chromosome alignment defects.

1099 **Immunofluorescence-based assessment of recombination kinetics**

1100 SC formation kinetics

1101 To assess SC formation we quantified SC formation in prophase stages that were identified
1102 based on axis morphology (Figure 3D and S5B). We focused on pre-mid pachytene stages in
1103 spermatocytes because autosomal SC defects cause spermatocyte elimination in mid pachytene
1104 (Burgoyne et al., 2009), which renders later stages of prophase unreliable for the quantification
1105 of autosomal SC defects in mutants with compromised synapsis. Hence, we quantified SC
1106 formation in juvenile mice before most spermatocytes reach mid pachytene, or we examined
1107 histone H1t (marker of post-mid pachytene stages) negative spermatocytes from adult (Figure
1108 3D). Quantification of SC formation has limitations. Reduction in the number of cells that
1109 completed SC formation between autosomes can reflect either a kinetic delay of SC formation
1110 and/or a terminal failure of SC formation in a subpopulation of early pachytene spermatocytes.
1111 Hence, it was not possible to judge if there was a significant terminal failure in SC formation or
1112 only a kinetic delay in *Ankrd31*^{-/-} spermatocytes.

1113 DSB repair focus kinetics

1114 To assess recombination kinetics we used immunostaining of DSB repair proteins that
1115 accumulate on the processed single-stranded DNA ends that result from DSBs (RPA, DMC1
1116 and RAD51). This type of analysis provides information about the steady state level of
1117 recombination intermediates that are marked by the respective proteins. Thus the number of foci
1118 could vary with both the formation kinetics and the turnover of intermediates. Hence, focus

1119 numbers do not directly reflect DSB numbers. Nonetheless, the numbers of RAD51/DMC1 foci
1120 are thought to reflect the numbers of unrepaired DNA ends that are available for homology
1121 search in the context of unsynapsed chromosome axes. RPA focus counts are thought to reflect
1122 the number of all the single-stranded DNA ends that are participating in recombination both in
1123 the context of unsynapsed and synapsed chromosome axes.

1124 **Gene expression analysis**

1125 In order to test the effect of *Ankrd31* deficiency in mouse testicular transcriptome total RNAs
1126 from *Ankrd31*^{+/+} and *Ankrd31*^{-/-} juvenile testes (12 days old) were extracted. RNA was extracted
1127 using RNeasy Mini (Qiagen, Cat No./ID: 74104) according to manufacturer's instructions.
1128 Quantification and quality control of RNA was performed using Agilent 2100 Bioanalyzer. mRNA
1129 was isolated from 300 ng total RNA by poly-dT enrichment followed by strand specific RNA-Seq
1130 library preparation (Ultra II Directional RNA Library Prep, NEB) following the manufacturer's
1131 instructions. Libraries were equimolarly pooled and subjected to 76 bp single end sequencing on
1132 a NextSeq 500 and Hiseq 2500 sequencer (Illumina) resulting in on average 29 Mio
1133 reads/sample (Bray et al., 2016; Kim et al., 2013).

1134 **Identification of Ankrd31 by transcriptome analysis**

1135 To screen for genes that are preferentially expressed in gonads, we profiled transcriptomes of
1136 embryonic female gonads, adult testes and an array of 17 somatic tissues from adult mice by
1137 RNA-sequencing of total RNAs. RNeasy Mini kit (Qiagen, Cat No./ID: 74104) was used
1138 according to manufacturer's instructions to purify total RNA from testes of adult mice and ovaries
1139 from fetal mice (C57/BL6xDBA/2 background). RNA was purified from ovaries that were pooled
1140 from 34-44 fetuses/newborn mice at six developmental timepoints, 11.5, 12.5, 14.5, 16.5, 18.5
1141 and 20.5 days post coitum. Total RNA samples from mouse somatic tissues were purchased via
1142 Ambion (liver, brain, thymus, heart, lung, spleen and kidney, Cat#7800) and Zyagen (mammary
1143 gland, pancreas, placenta, salivary gland, skeletal muscle, skin, small intestine, spinal cord,
1144 tongue and uterus, Cat#MR-010). Total RNA from the listed 17 somatic tissues were mixed in
1145 equal proportions to create a somatic RNA mix, and total RNAs from testes were mixed with this
1146 somatic RNA mix in 1:17 ratio to create a testis+somatic RNA mix. mRNAs were isolated from
1147 300 ng total RNA by poly-dT enrichment followed by strand specific RNA-Seq library preparation
1148 (Ultra II Directional RNA Library Prep, NEB) following the manufacturer's instructions. Libraries
1149 were subjected to 75 bp single end sequencing on a NextSeq 500 (Illumina) (Kim et al., 2013).

1150 We used the ovarian RNA-seq libraries as a starting point to identify previously unannotated
1151 protein-coding genes that were specifically expressed in reproductive tissues. This screen was

1152 based on the assumption that some of the meiosis-specific genes might have evaded
1153 annotation. To identify previously unrecognised transcripts, we used the Cufflinks suite of tools
1154 (Roberts et al., 2011) to assemble the mouse transcriptome *de novo* from our own RNA-seq
1155 data of gonads and published RNA-seq datasets of a comprehensive array of mouse tissues of
1156 the ENCODE project (Consortium, 2011, 2012)(data accessible at NCBI GEO database (Edgar
1157 et al., 2002), accession GSE36025). Tophat software was used to map reads to mouse genome
1158 assembly mm10 (Kim et al., 2013). We used Cufflinks (Roberts et al., 2011) to generate
1159 transcript contigs from mapped reads. Cuffmerge (Roberts et al., 2011) was used to merge the
1160 *de novo* assembled transcripts with the Ensembl 72 mouse transcriptome and to identify putative
1161 transcripts that were not present in the Ensembl 72 annotation. Fragment per kilobase per
1162 million (fkpm) counts were calculated for each transcript by the Cufflinks suite. The Emboss tool
1163 "getorf" was used to predict open reading frames (ORFs) in these putative transcripts (Rice et
1164 al., 2000), and InterProScan was used to identify conserved domains in the resulting predicted
1165 ORFs (Jones et al., 2014). We applied a complex set of criteria to identify genes that are
1166 potentially involved in processes related to meiotic recombination. First, we focused on
1167 transcripts whose expression peaked either in 14.5 or 16.5 days post coitum ovaries, and whose
1168 average expression was at least two times higher at these stages than in any other stage of
1169 ovary development. This criterion was chosen because leptotene to early pachytene stage
1170 oocytes, which are expected to express most of the genes involved in meiosis-specific aspects
1171 of recombination, are most abundant at 14.5 or 16.5 days post coitum. Second, we selected for
1172 transcripts whose maximum fkpm in ovaries was higher than 1, because transcripts of known
1173 meiosis-specific recombination proteins, e.g. Hormad1, Hormad2 or lho1, were expressed well
1174 above this level. Third, any transcript of interest had to have at least ten times higher fkpm
1175 values in the transcriptome of 8-week-old testis than in the transcriptome of any non-
1176 reproductive tissues from the ENCODE project (Consortium, 2011, 2012)(NCBI GEO accession
1177 GSE36025). This criterion ensured that we primarily identified transcripts that were specific to
1178 reproductive tissue where meiosis is ongoing. We identified only six transcript contigs that
1179 matched all three criteria. Three of these transcript contigs mapped to overlapping/adjacent
1180 genomic loci. These three transcripts contained ORFs that showed strong similarity to the ORF
1181 of the predicted human Ankrd31 gene. Only these three transcript contigs encoded predicted
1182 peptides with conserved domains. Since we were interested primarily in genes that were
1183 involved in conserved processes we selected the mouse equivalent of human *Ankrd31* for
1184 further functional analysis. We compared the full length human Ankrd31 transcript with our
1185 transcript contigs to define a predicted full length mouse Ankrd31 transcript and to define the
1186 exon structure of a predicted mouse Ankrd31 gene. Our prediction of a full length Ankrd31

1187 transcript closely matched the predicted mouse *Ankrd31* transcript (XM_006517797.1) of
1188 subsequent transcriptome annotations.

1189 To illustrate the expression of the consolidated full length *Ankrd31* transcript (Figure 1), we
1190 constructed a transcriptome index by using mm10 mouse reference transcriptome (Ensembl
1191 annotation version 87) and *gtf_to_fasta* from the TopHat2. TPM values were calculated by the
1192 software kallisto (Version 0.43.1) and the transcriptome index.

1193 **ChIP experiments**

1194 DMC1 ChIP-seq was performed as described in (Grey et al., 2017). Two testis from *Ankrd31*^{+/+}
1195 and *Ankrd31*^{-/-} from 6 weeks old mice were used for each replicate. Sequencing was performed
1196 on HiSeq 2500 Rapidmode 2x50b.

1197 H3K4me3 ChIP-seq was done as described in (Diagouraga et al., 2018). Four testis from
1198 *Ankrd31*^{+/+} and *Ankrd31*^{-/-} from 12dpp mice were used for each replicate. Sequencing was
1199 performed on HiSeq 2500 Rapidmode 1x75b.

1200 **ChIP-seq data computational analysis**

1201 Read alignment

1202 After quality control, H3K4me3 ChIP-seq and DMC1 ChIP-SSDS (Single Strand DNA
1203 Sequencing) reads were mapped to the UCSC mouse genome assembly build GRCm38/mm10.
1204 Bowtie2 (with default parameters) was used to map H3K4me3 ChIP-seq reads, while the
1205 previously published method (Khil et al., 2012) was used for DMC1 ChIP-SSDS reads (*i.e.* the
1206 BWA modified algorithm and a customized script, which were specifically developed to align and
1207 recover ssDNA fragments). A filtering step was then performed on aligned reads for all ChIP-seq
1208 experiments to keep non-duplicated and high-quality mapped reads with no more than one
1209 mismatch per read. When analyzing specifically the DMC1 and H3K4me3 signals covering the
1210 pseudo-autosomal region (PAR) of sex chromosome (Fig. 6F-G, Table S7), we used a read
1211 mapping performed over a customized genome which was depleted for the PAR from
1212 assembled chrY (*i.e.* only chrY:0-90745844 was included in this customized genome). This
1213 method ensured that all reads originated from either chrX- or chrY-PAR region were mapped
1214 over a unique reference sequence, preventing multimapping and thus misquantification over this
1215 region.

1216 Identifying meiotic hotspots

1217 To identify meiotic hotspots from biologically replicated samples in DMC1 ChIP-SSDS, we used
1218 the Irreproducible Discovery Rate (IDR) methodology, as previously described (Diagouraga et

1219 al., 2018). This method was developed for ChIP-seq analysis and extensively used by the
1220 ENCODE and modENCODE projects (Landt et al., 2012). The framework developed by Qunhua
1221 Li and Peter Bickel's group (<https://sites.google.com/site/anshulkundaje/projects/idr>) was
1222 followed. Briefly, this method allows testing the reproducibility within and between replicates by
1223 using the IDR statistics. Following their pipeline, peak calling was performed using MACS
1224 version 2.0.10 with relaxed conditions (--pvalue=0.1 --bw1000 --nomodel --shift400) on each of
1225 the two replicates, the pooled dataset, and on pseudo-replicates that were artificially generated
1226 by randomly sampling half of the reads twice for each replicate and the pooled dataset. Then
1227 IDR analyses were performed and reproducibility was checked. Final peak sets were built by
1228 selecting the top N peaks from pooled datasets (ranked by increasing p values), with N defined
1229 the highest value between n_1 (the number of overlapping peaks with an IDR below 0.01, when
1230 comparing pseudo replicates from pooled datasets) and n_2 (the number of overlapping peaks
1231 with an IDR below 0.05, when comparing the true replicates), as recommended for the mouse
1232 genome.

1233 Signal normalization and quantitative analysis

1234 All read distributions and signal intensities presented in this work were then calculated after
1235 pooling reads from both replicates. DMC1 ChIP-SSDS peaks were re-centered and read
1236 enrichment was normalized to the local background, as previously described (Brick et al., 2012).
1237 DMC1 ChIP-SSDS read enrichments could not be normalized between *Ankrd3^{+/+}* and *Ankrd31^{-/-}*
1238 mice. Indeed, the previously reported method to normalize such data (that is to consider that the
1239 total DMC1 signal should be the same between genotypes); (Davies et al., 2016; Diagouraga et
1240 al., 2018) cannot apply to our study as the DMC1 dynamics is altered in the mutant (see Figure
1241 4). Thus, only relative levels between regions or hotspots should be compared between different
1242 genotypes. SSDS counts from DMC1 ChIP are dependent on DMC1 half-life on ssDNA and are
1243 normalized to library size hence they can be only used to assess distribution but not the absolute
1244 numbers of single-stranded-DNA-containing recombination intermediates. The H3K4me3 signal
1245 recovered from young mice has been normalized between *Ankrd3^{+/+}* and *Ankrd31^{-/-}* mice
1246 assuming, as in previous studies (Davies et al., 2016; Diagouraga et al., 2018), that the signal of
1247 a PRDM9-independent set of H3K4me3 peaks should remain unchanged in testes of fertile mice
1248 at the same meiotic stage. Normalization factors were thus calculated based on the enrichment
1249 found at a set of 29 promoters of protein-coding genes (*Mlh1*, *Pms2*, *Mnd1*, *Dmc1*, *Rad21l*,
1250 *Mei4*, *Stra8*, *Ctcf1*, *Mei1*, *Puf60*, *Eef2*, *Rpl38*, *Leng8*, *Setx*, *Eif3f*, *Rpl37*, *Psmc4*, *Heatr3*,
1251 *Chmp2a*, *Sycp1*, *Sycp2*, *Sycp3*, *Morc2b*, *Zfp541*, *Spo11*, *Mdh1b*, *Rec8*, *Msh4*, *Psmc3ip*),
1252 suggested by Davies et al (2016). H3K4me3 signal presented in the present study were thus

1253 normalized by library size (Read per million of mapped reads; RPM) then to promoters to reach
1254 the level of *Ankrd31*^{+/+}.

1255 Determination of overlapping peaks

1256 DSB hotspots identified in *Ankrd31*^{-/-} mice were assigned to B6 or default hotspots on the basis
1257 of overlapping peak centers ± 200 bp. As reference for B6 hotspots we used previously published
1258 DSB maps (Diagouraga et al., 2018; Grey et al., 2017; Smagulova et al., 2016) and the DSB
1259 map of *Ankrd31*^{+/+} mice used in this study. As a reference for default DSB sites, we used the
1260 map of the *Prdm9*^{-/-} mice (Brick et al., 2012). When necessary, DSB hotspot coordinates were
1261 converted from GRCm37/mm9 to GRCm38/mm10 using the liftOver tool
1262 (<https://genome.ucsc.edu/cgi-bin/hgLiftOver>). Thus, 94% of peaks could be assigned to a known
1263 DSB hotspot, whereas a small fraction (6%) of DSB hotspots could not be assigned to any of the
1264 reference hotspots and was defined as new (Fig. 6B). These new hotspots have in average a
1265 weak SSDS signal (Fig. 6C) and could be PRDM9 dependent or independent.

1266 **ANKRD31 family collection and protein sequence analysis**

1267 A Hidden Markov Model (HMM) search with the mouse ANKRD31 protein in the PFAM database
1268 (v. 31.0, March 2017, (Finn et al., 2016)) detected two ankyrin repeat regions, with 3 copies
1269 each (amino acids 467-568 and 1167-1279) with highly significant E-values (1.6e-35). To search
1270 for orthologs, we performed a NCBI blastp search within the complete set of UniProt reference
1271 proteomes or the NCBI non-redundant protein database, restricting the query to the region c-
1272 terminal of the last ankyrin repeat (amino acids 1280-1856) (Sayers et al., 2011). The Ankrd31
1273 protein family is well conserved within vertebrates and orthologs can be collected in reciprocal
1274 blasts by applying highly significant e-values (1e-10). For a multiple alignment, sequences were
1275 derived either from the UniProt database, as for the Ankrd31 orthologs of Homo sapiens
1276 (sp|Q8N7Z5|ANR31_HUMAN), Ornithorhynchus anatinus (tr|F7FXW5|F7FXW5_ORNAN), and
1277 Xenopus tropicalis (tr|F6ZDM1|F6ZDM1_XENTR), or from the NCBI protein database, as for
1278 Canis lupus familiaris (ref|XP_022272501.1|), Mus musculus (ref|XP_006517860.1|), Columba
1279 livia (ref|XP_021147901.1|), Chrysemys picta bellii (ref|XP_023960269.1|), Struthio camelus
1280 australis (ref|XP_009686903.1|), Callorhinchus milii (ref|XP_007903536.1|), and Takifugu
1281 rubripes (ref|XP_011613543.1|). Multiple alignment was performed with MAFFT (-linsi v7.313,
1282 (Kato and Toh, 2008)), and visualized with ClustalX (v2.1,(Thompson et al., 1997)). 5
1283 conserved regions were identified. The first ankyrin repeat region is missing in bony fish, but
1284 present in shark, whereas the second ankyrin repeat region is highly conserved in all vertebrate
1285 orthologs. The third conserved region CR3 includes a predicted coiled coil interaction domain (in

1286 mouse ANKRD31 from 1340 to 1374 (Lupas et al., 1991)). For the conserved regions CR4
1287 (1701-1787) and CR5 (1811-1857) no function could be assigned.

1288

1289 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1290 Statistical analysis of cytological observations was done by GrapPad Prism 7. All other statistical
1291 tests were done using R version 3.3.3, if not otherwise stated. All tests and p-values are
1292 provided in the corresponding legends and/or figures.

1293

1294 **DATA AND SOFTWARE AVAILABILITY**

1295 RNA-seq and CHIP-seq data have been deposited in NCBI's Gene Expression Omnibus (Edgar
1296 et al., 2002) and are accessible through GEO Series accession numbers GSE119410,
1297 GSE119411 and GSE126443 at the "<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=>"
1298 address. Raw data for figures has been deposited in Mendeley Database:
1299 <http://dx.doi.org/10.17632/jfb3msz44d.1>

1300

1301

1302 **References**

- 1303 Acquaviva, L., Boekhout, M., Karasu, M.E., Brick, K., Pratto, F., van Overbeek, M., Kauppi, L., Camerini-
1304 Otero, R.D., Jasin, M., and Keeney, S. (2019). Ensuring meiotic DNA break formation in the mouse
1305 pseudoautosomal region. In bioRxiv.
- 1306 Ahmed, E.A., and de Rooij, D.G. (2009). Staging of mouse seminiferous tubule cross-sections. *Methods*
1307 *Mol Biol* 558, 263-277.
- 1308 Barchi, M., Mahadevaiah, S., Di Giacomo, M., Baudat, F., de Rooij, D.G., Burgoyne, P.S., Jasin, M., and
1309 Keeney, S. (2005). Surveillance of different recombination defects in mouse spermatocytes yields distinct
1310 responses despite elimination at an identical developmental stage. *Molecular and cellular biology* 25,
1311 7203-7215.
- 1312 Barchi, M., Roig, I., Di Giacomo, M., de Rooij, D.G., Keeney, S., and Jasin, M. (2008). ATM promotes the
1313 obligate XY crossover and both crossover control and chromosome axis integrity on autosomes. *PLoS*
1314 *genetics* 4, e1000076.
- 1315 Bellani, M.A., Romanienko, P.J., Cairatti, D.A., and Camerini-Otero, R.D. (2005). SPO11 is required for sex-
1316 body formation, and *Spo11* heterozygosity rescues the prophase arrest of *Atm*^{-/-} spermatocytes. *J Cell*
1317 *Sci* 118, 3233-3245.
- 1318 Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P.C., Nicolas, A., and Forterre, P. (1997). An atypical
1319 topoisomerase II from Archaea with implications for meiotic recombination. *Nature* 386, 414-417.
- 1320 Blat, Y., Protacio, R.U., Hunter, N., and Kleckner, N. (2002). Physical and functional interactions among
1321 basic chromosome organizational features govern early steps of meiotic chiasma formation. *Cell* 111,
1322 791-802.

1323 Bray, N.L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic RNA-seq
1324 quantification. *Nature biotechnology* 34, 525-527.

1325 Brick, K., Smagulova, F., Khil, P., Camerini-Otero, R.D., and Petukhova, G.V. (2012). Genetic
1326 recombination is directed away from functional genomic elements in mice. *Nature* 485, 642-645.

1327 Buard, J., Barthes, P., Grey, C., and de Massy, B. (2009). Distinct histone modifications define initiation
1328 and repair of meiotic recombination in the mouse. *The EMBO journal* 28, 2616-2624.

1329 Burgoyne, P.S., Mahadevaiah, S.K., and Turner, J.M. (2009). The consequences of asynapsis for
1330 mammalian meiosis. *Nat Rev Genet* 10, 207-216.

1331 Chicheportiche, A., Bernardino-Sgherri, J., de Massy, B., and Dutrillaux, B. (2007). Characterization of
1332 Spo11-dependent and independent phospho-H2AX foci during meiotic prophase I in the male mouse. *J*
1333 *Cell Sci* 120, 1733-1742.

1334 Consortium, E.P. (2011). A user's guide to the encyclopedia of DNA elements (ENCODE). *PLoS biology* 9,
1335 e1001046.

1336 Consortium, E.P. (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature*
1337 489, 57-74.

1338 Daniel, K., Lange, J., Hached, K., Fu, J., Anastassiadis, K., Roig, I., Cooke, H.J., Stewart, A.F., Wassmann, K.,
1339 Jasin, M., *et al.* (2011). Meiotic homologue alignment and its quality surveillance are controlled by mouse
1340 *HORMAD1*. *Nature Cell Biology* 13, 599-U232.

1341 Davies, B., Hatton, E., Altemose, N., Hussin, J.G., Pratto, F., Zhang, G., Hinch, A.G., Moralli, D., Biggs, D.,
1342 Diaz, R., *et al.* (2016). Re-engineering the zinc fingers of PRDM9 reverses hybrid sterility in mice. *Nature*
1343 530, 171-176.

1344 de Massy, B. (2013). Initiation of meiotic recombination: how and where? Conservation and specificities
1345 among eukaryotes. *Annual review of genetics* 47, 563-599.

1346 Di Giacomo, M., Barchi, M., Baudat, F., Edelmann, W., Keeney, S., and Jasin, M. (2005). Distinct DNA-
1347 damage-dependent and -independent responses drive the loss of oocytes in recombination-defective
1348 mouse mutants. *Proc Natl Acad Sci U S A* 102, 737-742.

1349 Diagouraga, B., Clement, J.A.J., Duret, L., Kadlec, J., de Massy, B., and Baudat, F. (2018). PRDM9
1350 Methyltransferase Activity Is Essential for Meiotic DNA Double-Strand Break Formation at Its Binding
1351 Sites. *Mol Cell* 69, 853-865 e856.

1352 Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and
1353 hybridization array data repository. *Nucleic acids research* 30, 207-210.

1354 Enders, G.C., and May, J.J., 2nd (1994). Developmentally regulated expression of a mouse germ cell
1355 nuclear antigen examined from embryonic day 11 to adult in male and female mice. *Developmental*
1356 *biology* 163, 331-340.

1357 Finn, R.D., Coghill, P., Eberhardt, R.Y., Eddy, S.R., Mistry, J., Mitchell, A.L., Potter, S.C., Punta, M., Qureshi,
1358 M., Sangrador-Vegas, A., *et al.* (2016). The Pfam protein families database: towards a more sustainable
1359 future. *Nucleic acids research* 44, D279-285.

1360 Ghafari, F., Gutierrez, C.G., and Hartshorne, G.M. (2007). Apoptosis in mouse fetal and neonatal oocytes
1361 during meiotic prophase one. *BMC developmental biology* 7, 87.

1362 Grey, C., Baudat, F., and de Massy, B. (2018). PRDM9, a driver of the genetic map. *PLoS genetics* 14,
1363 e1007479.

1364 Grey, C., Clement, J.A., Buard, J., Leblanc, B., Gut, I., Gut, M., Duret, L., and de Massy, B. (2017). In vivo
1365 binding of PRDM9 reveals interactions with noncanonical genomic sites. *Genome research* 27, 580-590.

1366 Hayashi, K., Yoshida, K., and Matsui, Y. (2005). A histone H3 methyltransferase controls epigenetic events
1367 required for meiotic prophase. *Nature* 438, 374-378.

1368 Holloway, J.K., Morelli, M.A., Borst, P.L., and Cohen, P.E. (2010). Mammalian BLM helicase is critical for
1369 integrating multiple pathways of meiotic recombination. *J Cell Biol* 188, 779-789.

1370 Hunter, N. (2015). Meiotic Recombination: The Essence of Heredity. *Cold Spring Harbor perspectives in*
1371 *biology* 7.

1372 Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.R., and Joung,
1373 J.K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature biotechnology* *31*,
1374 227-229.

1375 Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A.,
1376 Nuka, G., *et al.* (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* *30*,
1377 1236-1240.

1378 Katoh, K., and Toh, H. (2008). Recent developments in the MAFFT multiple sequence alignment program.
1379 *Briefings in bioinformatics* *9*, 286-298.

1380 Kauppi, L., Barchi, M., Baudat, F., Romanienko, P.J., Keeney, S., and Jasin, M. (2011). Distinct properties
1381 of the XY pseudoautosomal region crucial for male meiosis. *Science* *331*, 916-920.

1382 Kauppi, L., Barchi, M., Lange, J., Baudat, F., Jasin, M., and Keeney, S. (2013). Numerical constraints and
1383 feedback control of double-strand breaks in mouse meiosis. *Genes & development* *27*, 873-886.

1384 Keeney, S., Giroux, C.N., and Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are
1385 catalyzed by Spo11, a member of a widely conserved protein family. *Cell* *88*, 375-384.

1386 Keeney, S., Lange, J., and Mohibullah, N. (2014). Self-organization of meiotic recombination initiation:
1387 general principles and molecular pathways. *Annual review of genetics* *48*, 187-214.

1388 Khil, P.P., Smagulova, F., Brick, K.M., Camerini-Otero, R.D., and Petukhova, G.V. (2012). Sensitive
1389 mapping of recombination hotspots using sequencing-based detection of ssDNA. *Genome research* *22*,
1390 957-965.

1391 Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate
1392 alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome biology*
1393 *14*, R36.

1394 Kumar, R., Bourbon, H.M., and de Massy, B. (2010). Functional conservation of Mei4 for meiotic DNA
1395 double-strand break formation from yeasts to mice. *Genes & development* *24*, 1266-1280.

1396 Kumar, R., Ghyselink, N., Ishiguro, K.I., Watanabe, Y., Kouznetsova, A., Hoog, C., Strong, E., Schimenti, J.,
1397 Daniel, K., Toth, A., *et al.* (2015). MEI4: a central player in the regulation of meiotic DNA double strand
1398 break formation in the mouse. *J Cell Sci.*

1399 Kumar, R., Oliver, C., Brun, C., Juarez-Martinez, A.B., Tarabay, Y., Kadlec, J., and de Massy, B. (2018).
1400 Mouse REC114 is essential for meiotic DNA double-strand break formation and forms a complex with
1401 MEI4. *Life Sci Alliance* *1*, e201800259.

1402 Landt, S.G., Marinov, G.K., Kundaje, A., Kheradpour, P., Pauli, F., Batzoglou, S., Bernstein, B.E., Bickel, P.,
1403 Brown, J.B., Cayting, P., *et al.* (2012). ChIP-seq guidelines and practices of the ENCODE and modENCODE
1404 consortia. *Genome research* *22*, 1813-1831.

1405 Lange, J., Yamada, S., Tischfield, S.E., Pan, J., Kim, S., Zhu, X., Socci, N.D., Jasin, M., and Keeney, S. (2016).
1406 The Landscape of Mouse Meiotic Double-Strand Break Formation, Processing, and Repair. *Cell* *167*, 695-
1407 708 e616.

1408 Latrille, T., Duret, L., and Lartillot, N. (2017). The Red Queen model of recombination hot-spot evolution:
1409 a theoretical investigation. *Philosophical transactions of the Royal Society of London. Series B, Biological*
1410 *sciences* *372*.

1411 Lesecque, Y., Glemin, S., Lartillot, N., Mouchiroud, D., and Duret, L. (2014). The red queen model of
1412 recombination hotspots evolution in the light of archaic and modern human genomes. *PLoS genetics* *10*,
1413 e1004790.

1414 Lupas, A., Van Dyke, M., and Stock, J. (1991). Predicting coiled coils from protein sequences. *Science* *252*,
1415 1162-1164.

1416 Mosavi, L.K., Cammett, T.J., Desrosiers, D.C., and Peng, Z.Y. (2004). The ankyrin repeat as molecular
1417 architecture for protein recognition. *Protein science : a publication of the Protein Society* *13*, 1435-1448.

1418 Myers, S., Bowden, R., Tumian, A., Bontrop, R.E., Freeman, C., MacFie, T.S., McVean, G., and Donnelly, P.
1419 (2010). Drive against hotspot motifs in primates implicates the PRDM9 gene in meiotic recombination.
1420 *Science* *327*, 876-879.

1421 Page, J., de la Fuente, R., Manterola, M., Parra, M.T., Viera, A., Berríos, S., Fernández-Donoso, R., and
1422 Rufas, J.S. (2012). Inactivation or non-reactivation: what accounts better for the silence of sex
1423 chromosomes during mammalian male meiosis? *Chromosoma* 121, 307-326.

1424 Panizza, S., Mendoza, M.A., Berlinger, M., Huang, L., Nicolas, A., Shirahige, K., and Klein, F. (2011). Spo11-
1425 accessory proteins link double-strand break sites to the chromosome axis in early meiotic recombination.
1426 *Cell* 146, 372-383.

1427 Peters, A.H., Plug, A.W., van Vugt, M.J., and de Boer, P. (1997). A drying-down technique for the
1428 spreading of mammalian meiocytes from the male and female germline. *Chromosome Res* 5, 66-68.

1429 Powers, N.R., Parvanov, E.D., Baker, C.L., Walker, M., Petkov, P.M., and Paigen, K. (2016). The Meiotic
1430 Recombination Activator PRDM9 Trimethylates Both H3K36 and H3K4 at Recombination Hotspots In
1431 Vivo. *PLoS genetics* 12, e1006146.

1432 Pratto, F., Brick, K., Khil, P., Smagulova, F., Petukhova, G.V., and Camerini-Otero, R.D. (2014). DNA
1433 recombination. Recombination initiation maps of individual human genomes. *Science* 346, 1256442.

1434 Rice, P., Longden, I., and Bleasby, A. (2000). EMBOSS: the European Molecular Biology Open Software
1435 Suite. *Trends in genetics : TIG* 16, 276-277.

1436 Robert, T., Nore, A., Brun, C., Maffre, C., Crimi, B., Bourbon, H.M., and de Massy, B. (2016). The TopoVIB-
1437 Like protein family is required for meiotic DNA double-strand break formation. *Science* 351, 943-949.

1438 Roberts, A., Pimentel, H., Trapnell, C., and Pachter, L. (2011). Identification of novel transcripts in
1439 annotated genomes using RNA-Seq. *Bioinformatics* 27, 2325-2329.

1440 Royo, H., Prosser, H., Ruzankina, Y., Mahadevaiah, S.K., Cloutier, J.M., Baumann, M., Fukuda, T., Hoog, C.,
1441 Toth, A., de Rooij, D.G., *et al.* (2013). ATR acts stage specifically to regulate multiple aspects of
1442 mammalian meiotic silencing. *Genes & development* 27, 1484-1494.

1443 Sarbajna, S., Denniff, M., Jeffreys, A.J., Neumann, R., Soler Artigas, M., Veselis, A., and May, C.A. (2012).
1444 A major recombination hotspot in the XqYq pseudoautosomal region gives new insight into processing of
1445 human gene conversion events. *Human molecular genetics* 21, 2029-2038.

1446 Sayers, E.W., Barrett, T., Benson, D.A., Bolton, E., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M.,
1447 DiCuccio, M., Federhen, S., *et al.* (2011). Database resources of the National Center for Biotechnology
1448 Information. *Nucleic acids research* 39, D38-51.

1449 Smagulova, F., Brick, K., Pu, Y., Camerini-Otero, R.D., and Petukhova, G.V. (2016). The evolutionary
1450 turnover of recombination hot spots contributes to speciation in mice. *Genes & development* 30, 266-
1451 280.

1452 Smagulova, F., Gregoret, I.V., Brick, K., Khil, P., Camerini-Otero, R.D., and Petukhova, G.V. (2011).
1453 Genome-wide analysis reveals novel molecular features of mouse recombination hotspots. *Nature* 472,
1454 375-378.

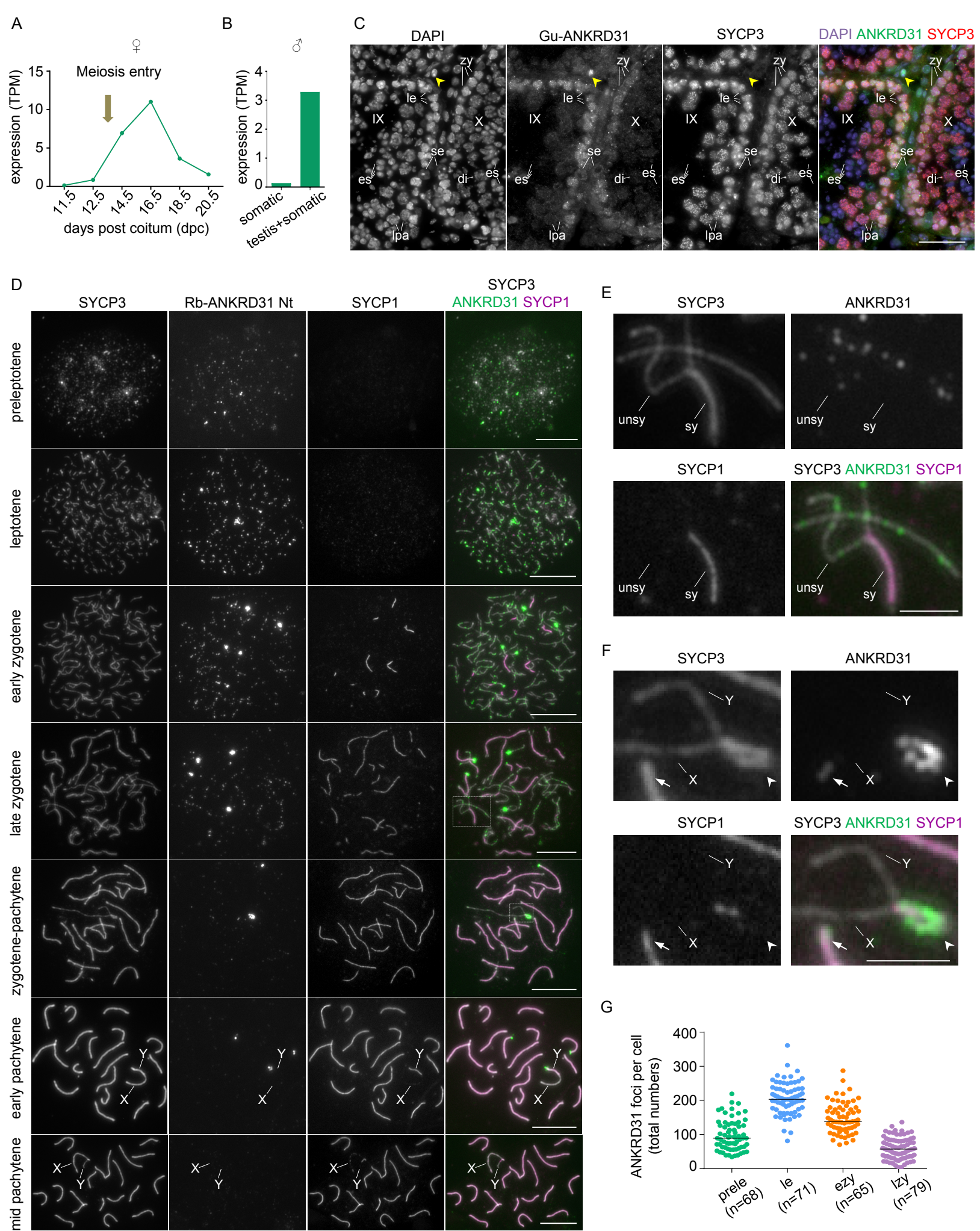
1455 Stanzione, M., Baumann, M., Papanikos, F., Dereli, I., Lange, J., Ramlal, A., Trankner, D., Shibuya, H., de
1456 Massy, B., Watanabe, Y., *et al.* (2016). Meiotic DNA break formation requires the unsynapsed
1457 chromosome axis-binding protein IHO1 (CCDC36) in mice. *Nat Cell Biol* 18, 1208-1220.

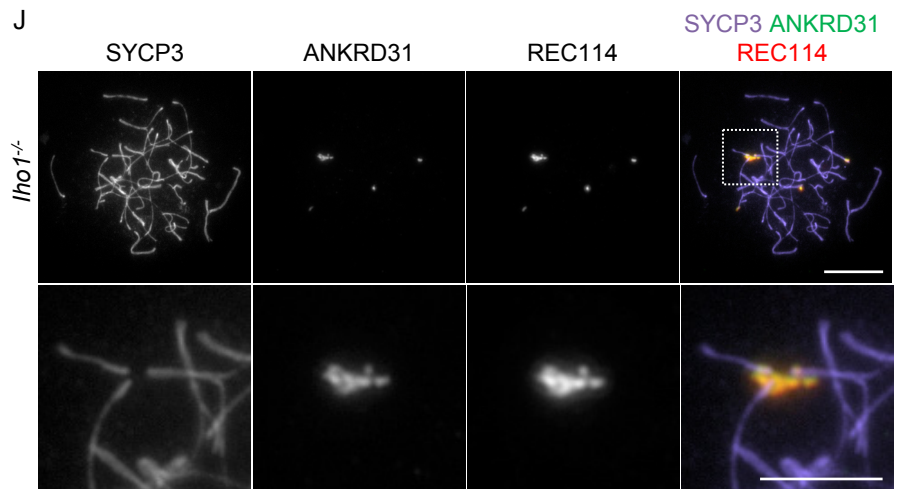
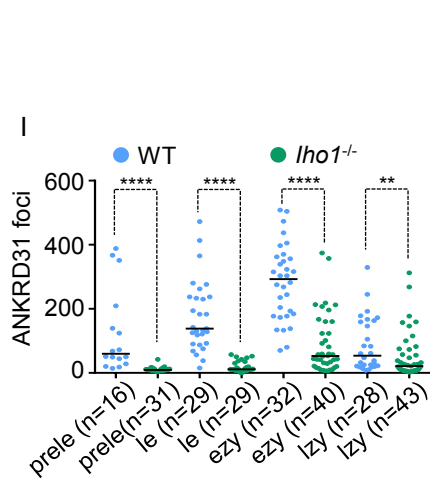
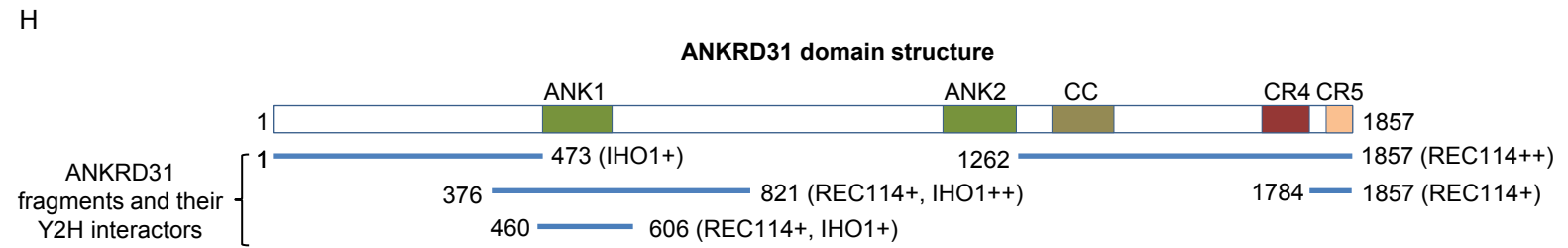
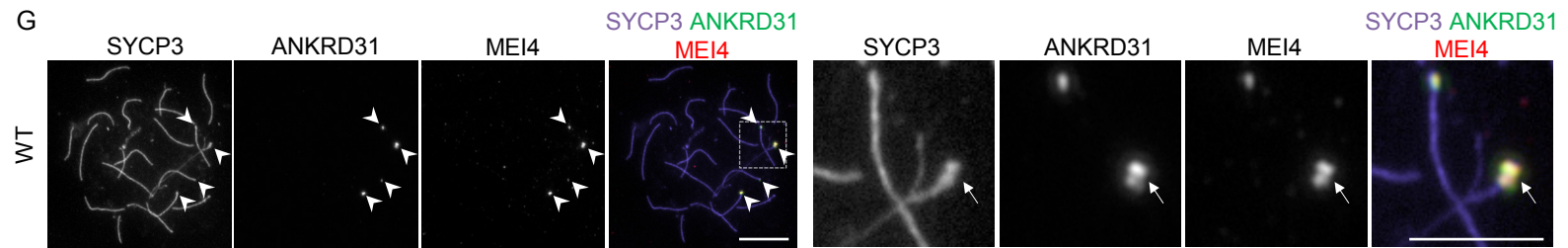
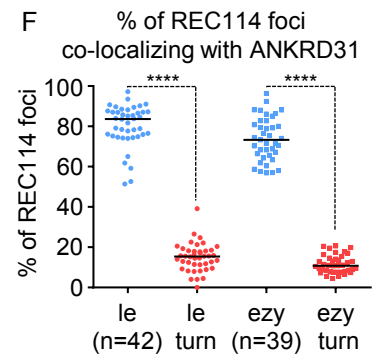
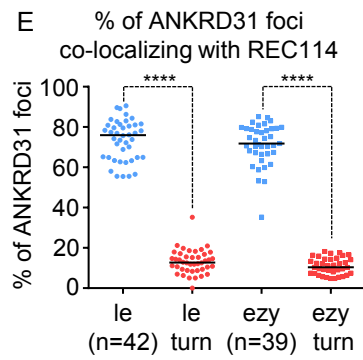
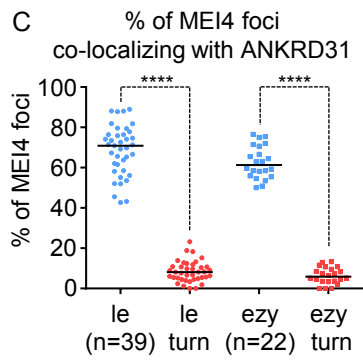
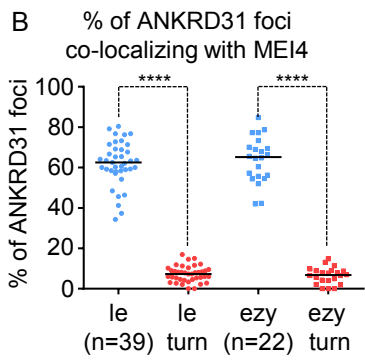
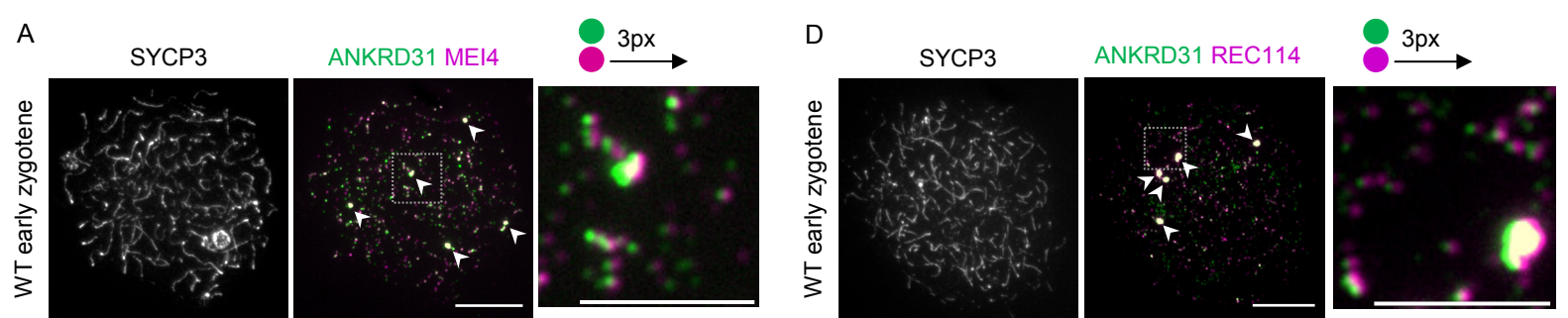
1458 Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997). The CLUSTAL_X
1459 windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools.
1460 *Nucleic acids research* 25, 4876-4882.

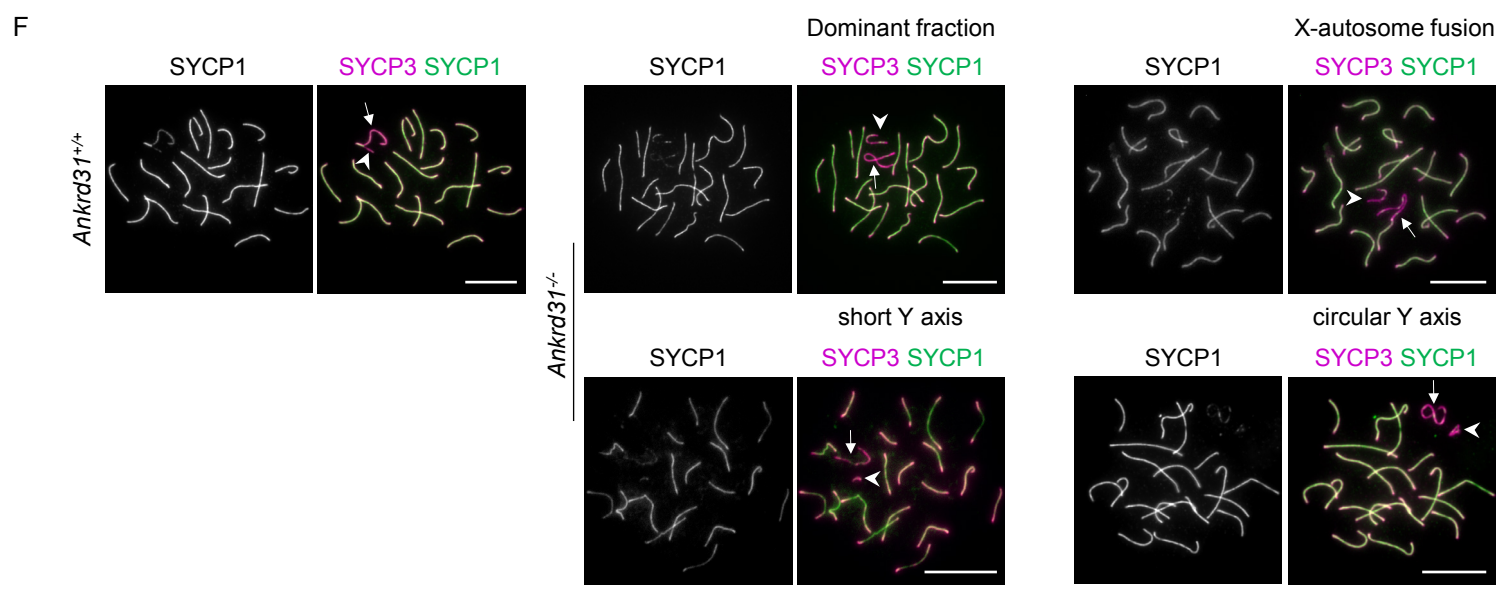
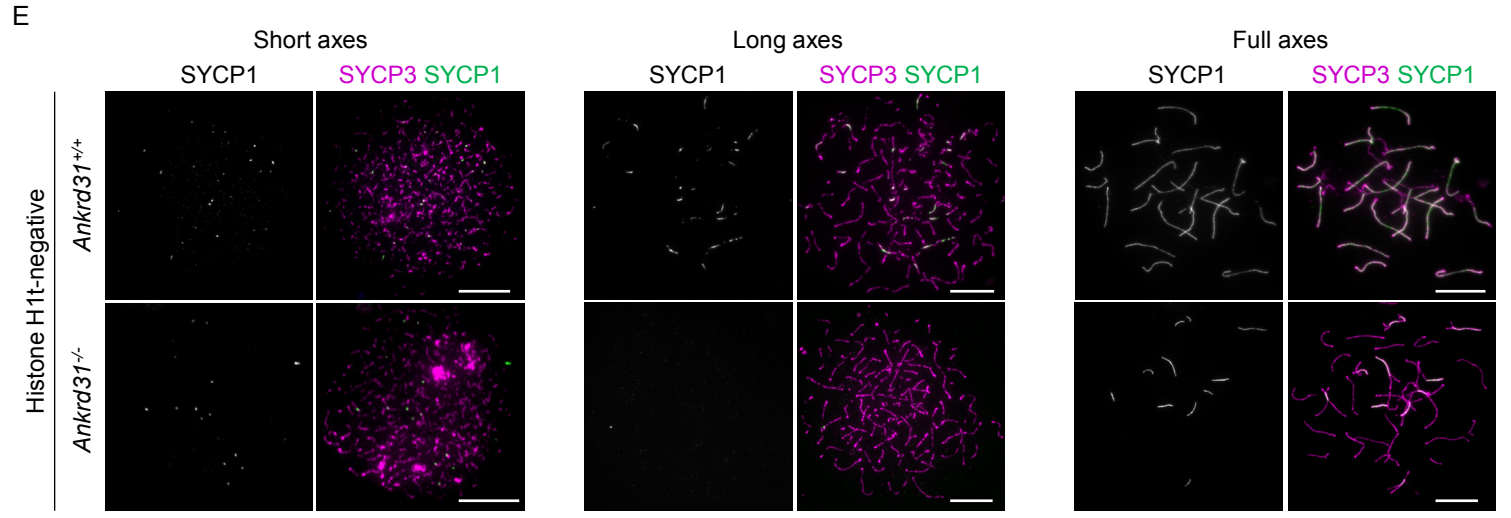
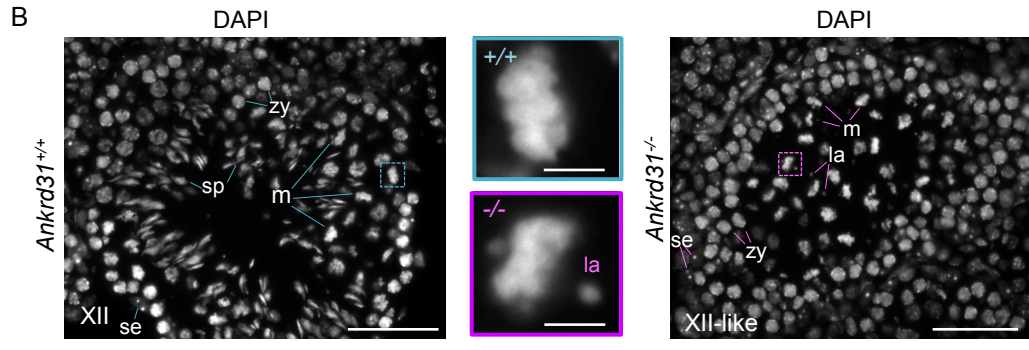
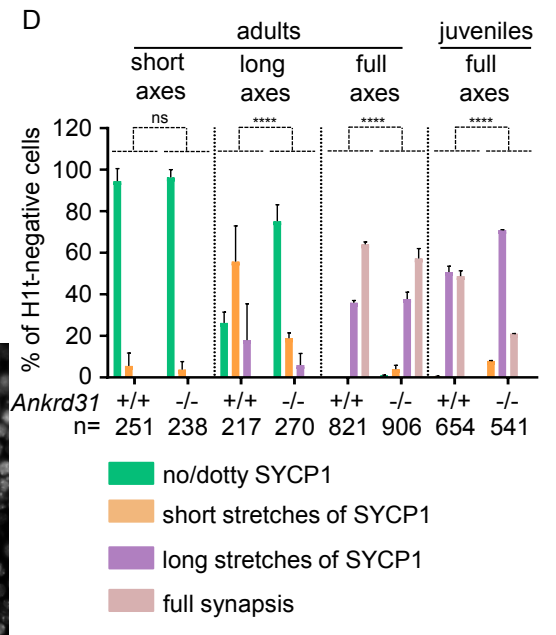
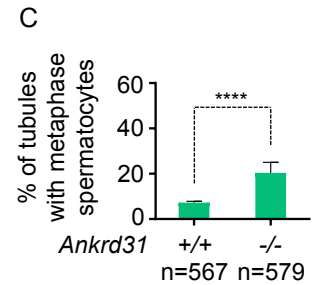
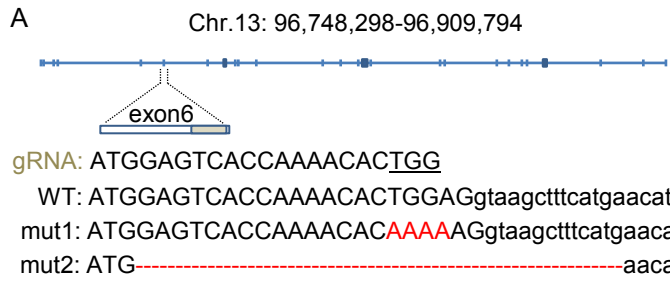
1461 Vrielynck, N., Chambon, A., Vezon, D., Pereira, L., Chelysheva, L., De Muyt, A., Mezard, C., Mayer, C., and
1462 Grelon, M. (2016). A DNA topoisomerase VI-like complex initiates meiotic recombination. *Science* 351,
1463 939-943.

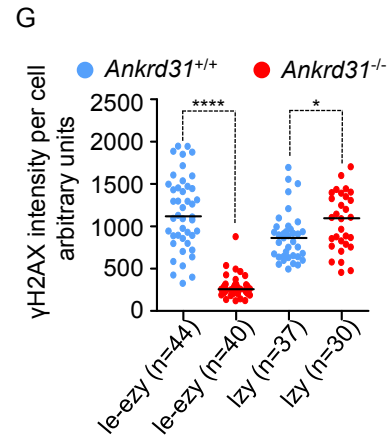
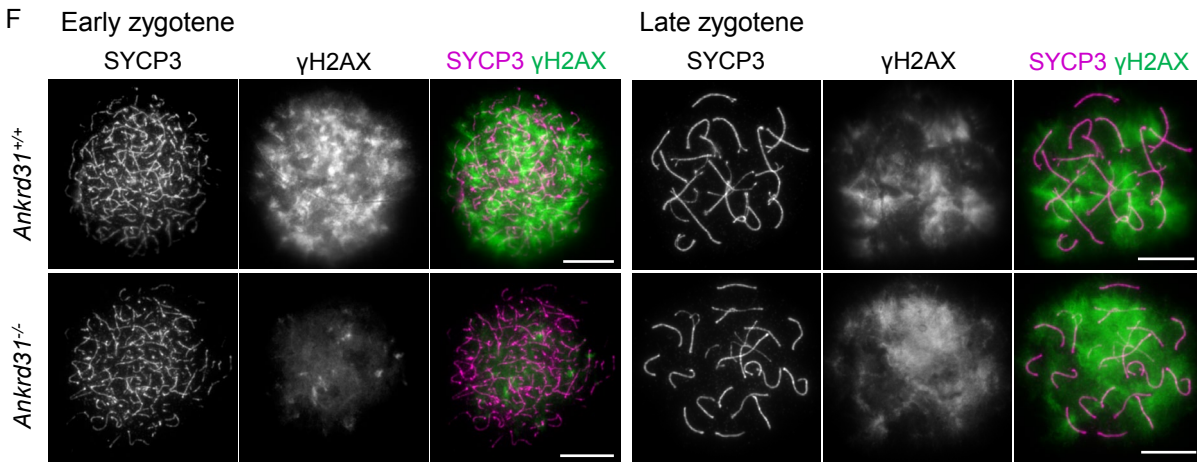
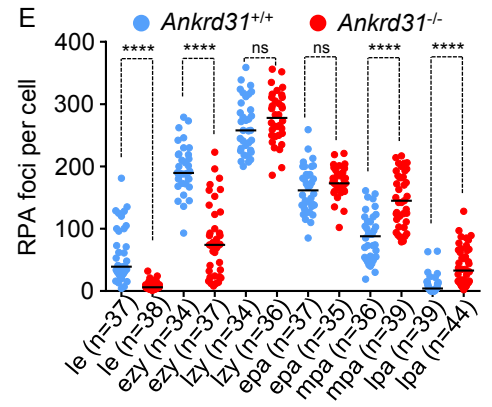
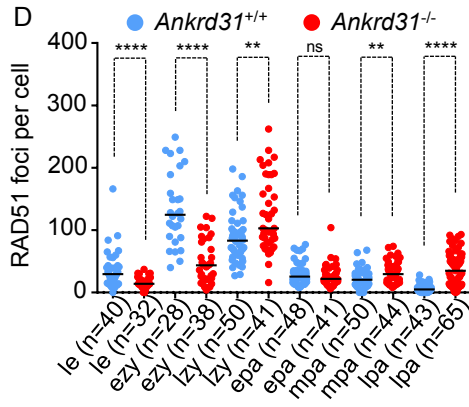
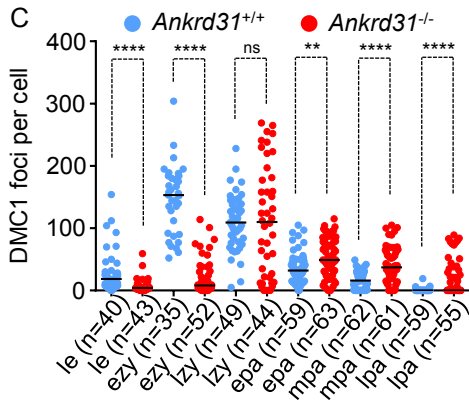
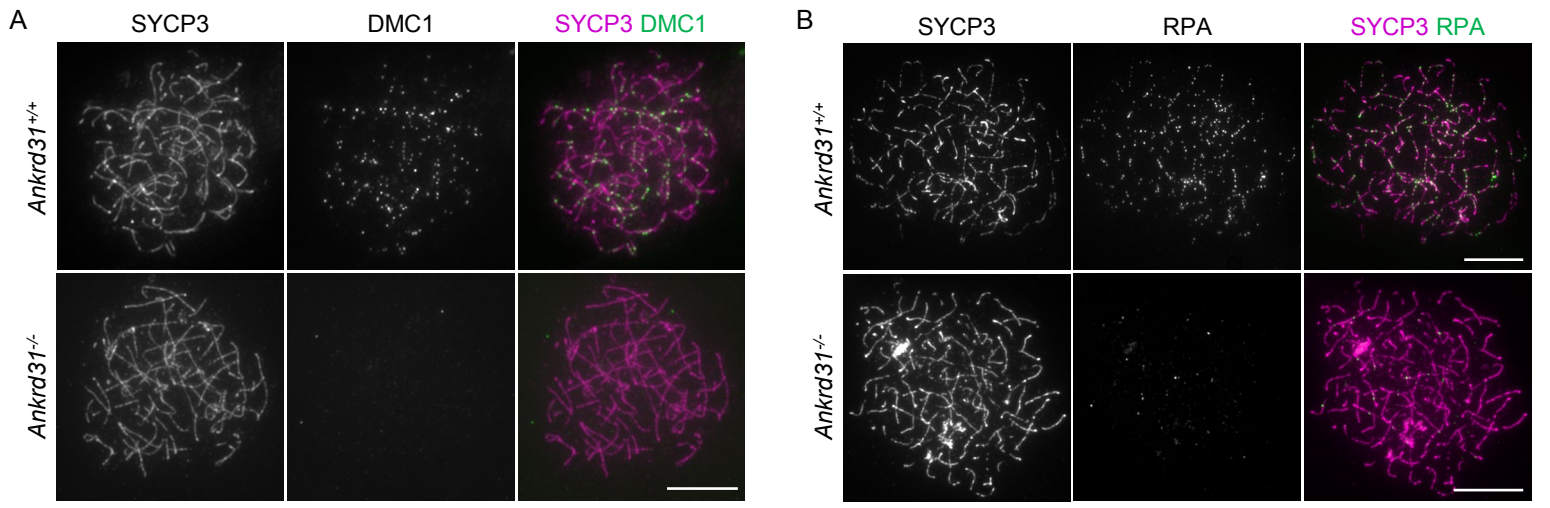
1464 Wojtasz, L., Daniel, K., Roig, I., Bolcun-Filas, E., Xu, H.L., Boonsanay, V., Eckmann, C.R., Cooke, H.J., Jasin,
1465 M., Keeney, S., *et al.* (2009). Mouse HORMAD1 and HORMAD2, Two Conserved Meiotic Chromosomal
1466 Proteins, Are Depleted from Synapsed Chromosome Axes with the Help of TRIP13 AAA-ATPase. *PLoS*
1467 *genetics* 5.

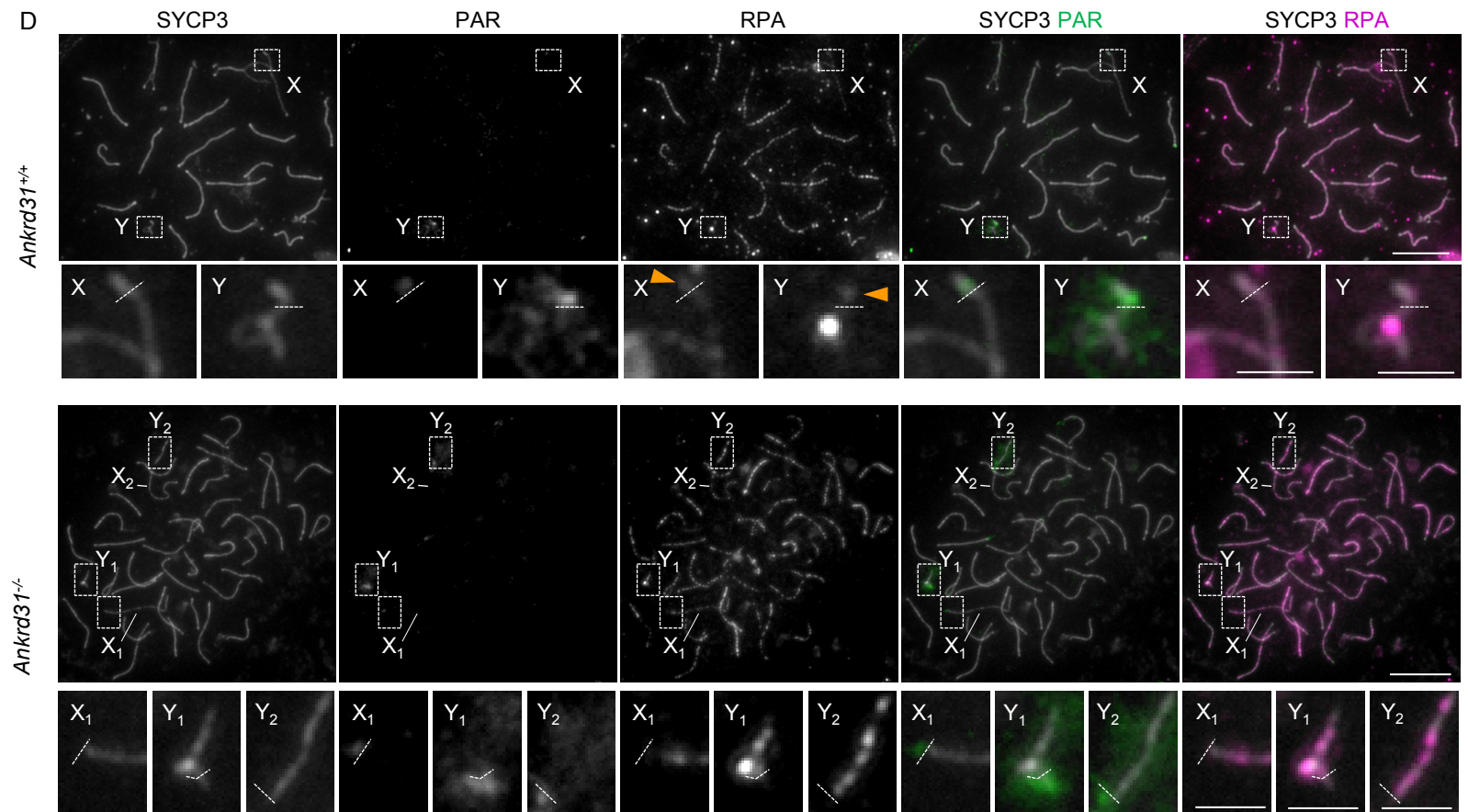
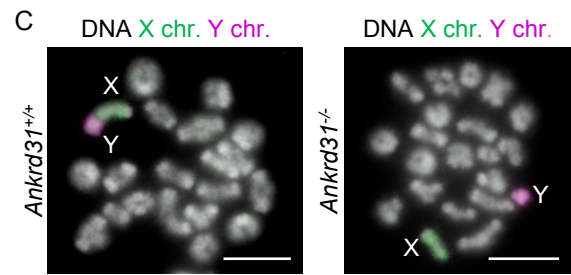
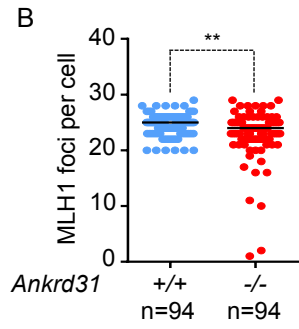
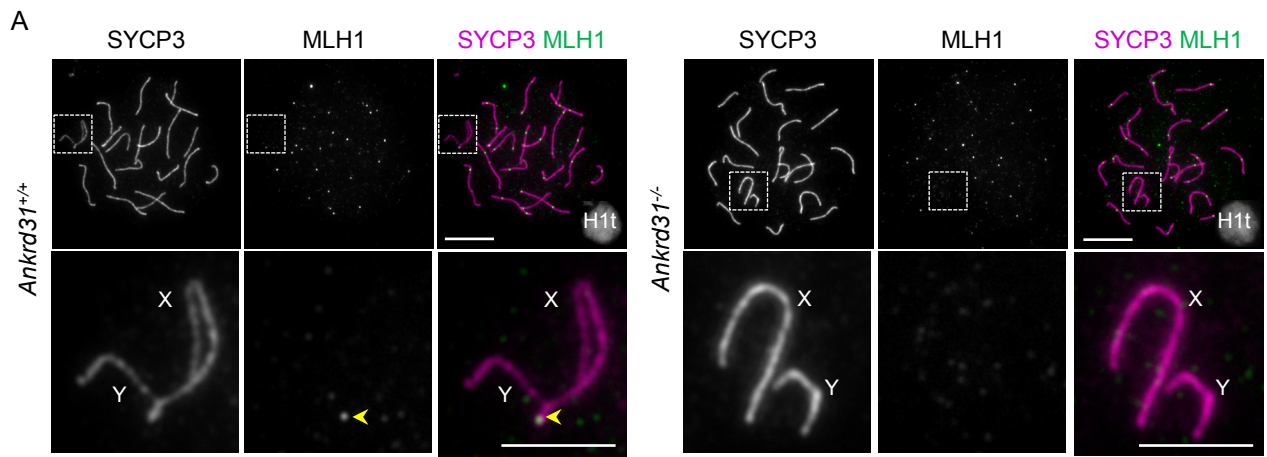
1468 Zickler, D., and Kleckner, N. (1999). Meiotic chromosomes: integrating structure and function. *Annual*
1469 *review of genetics* 33, 603-754.

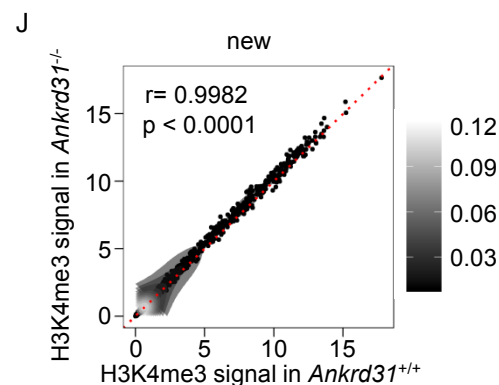
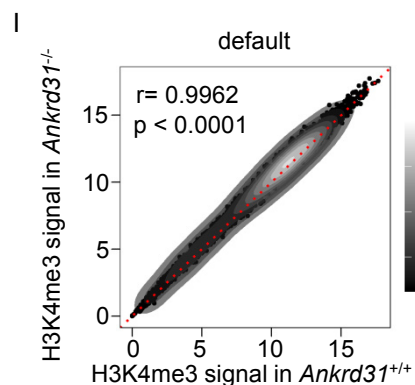
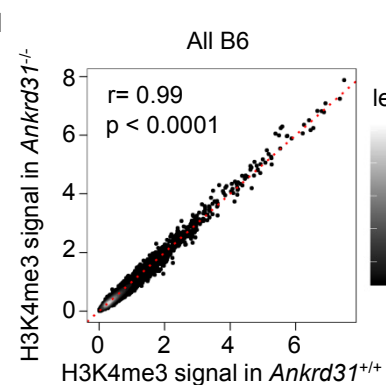
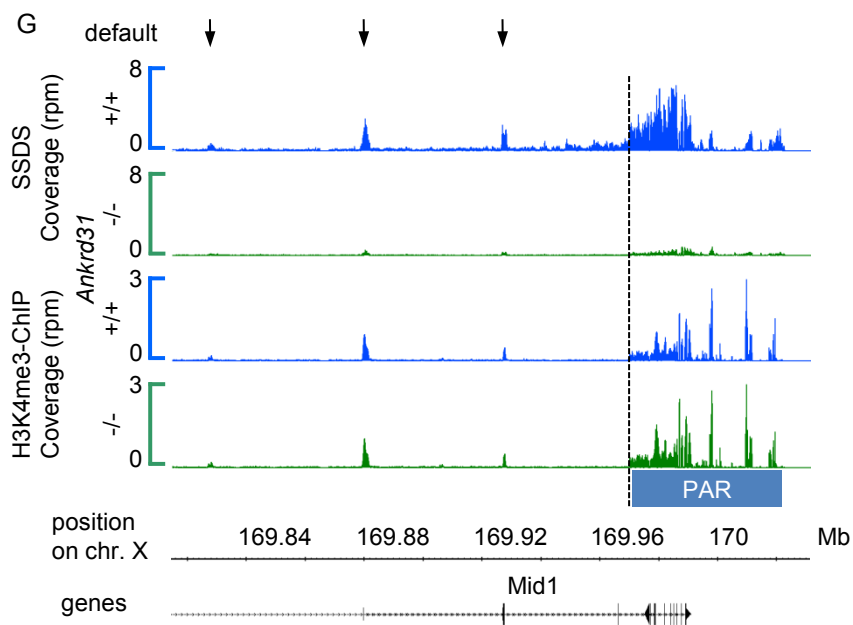
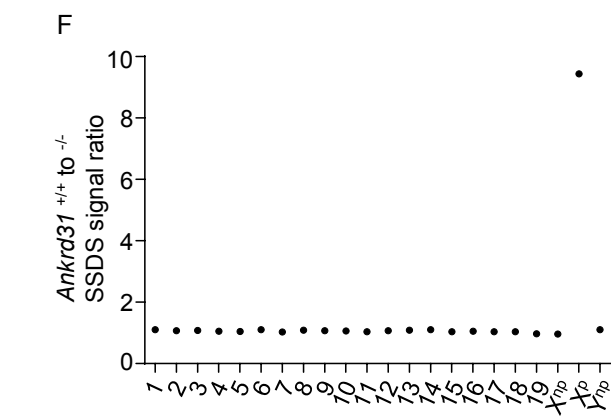
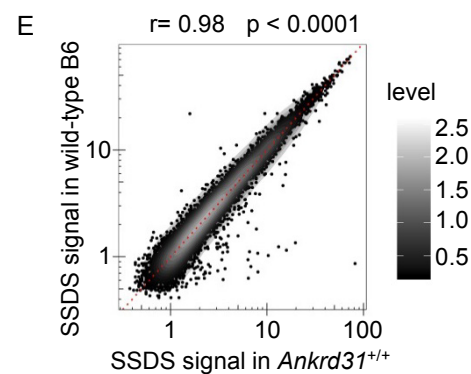
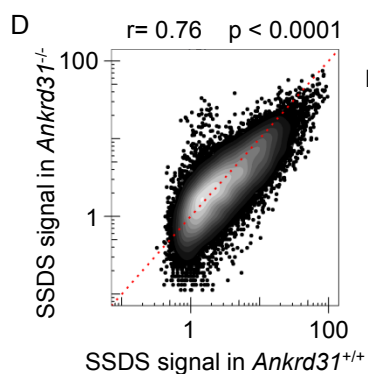
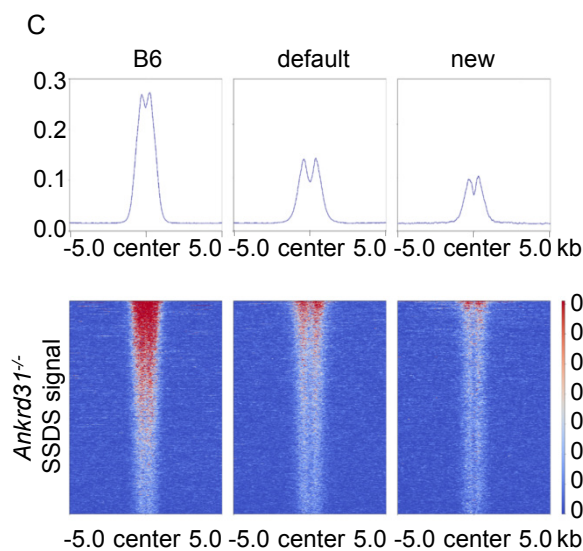
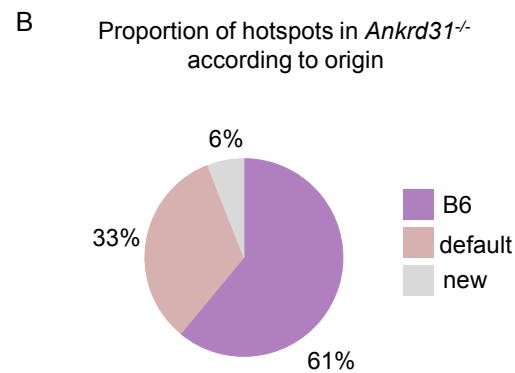
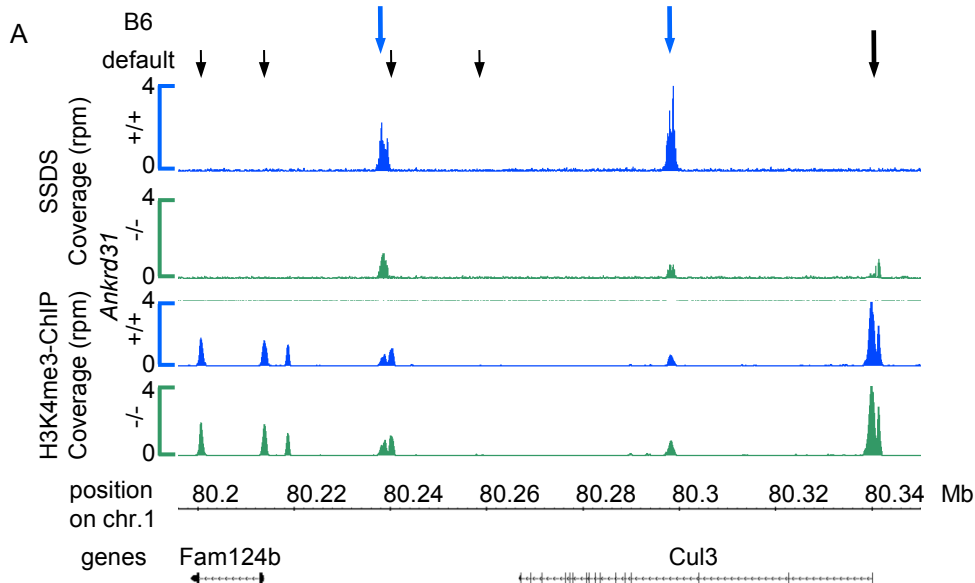


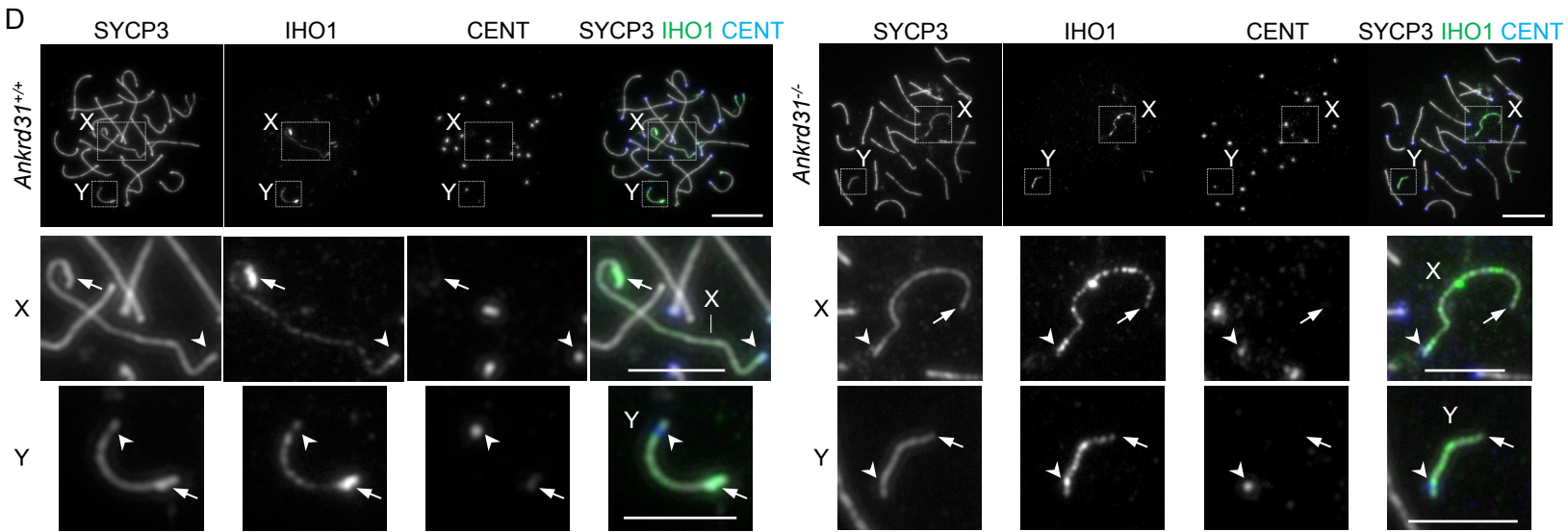
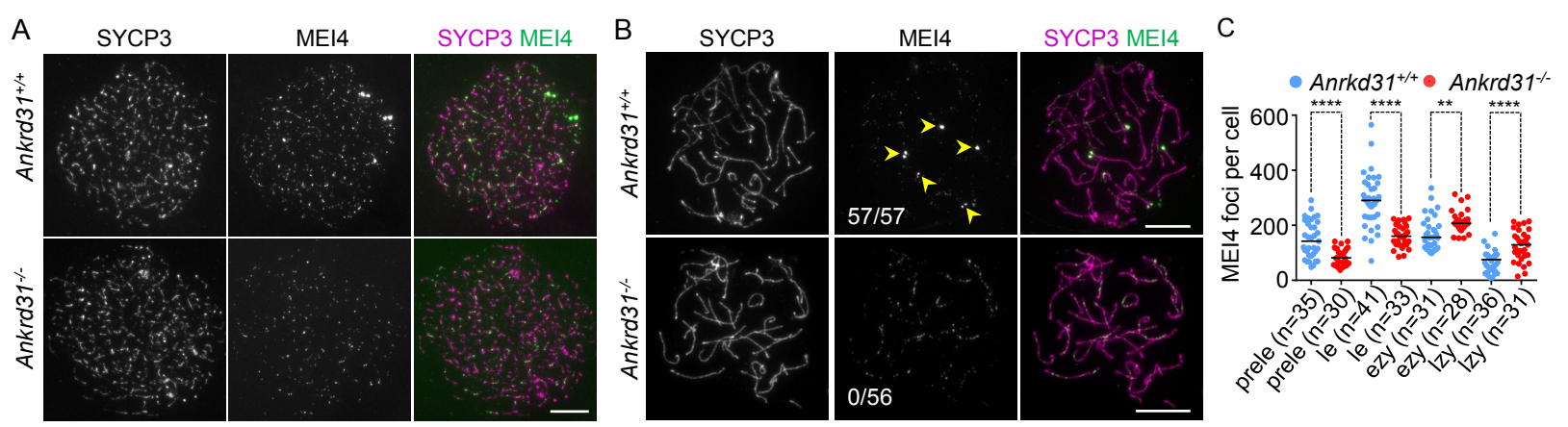








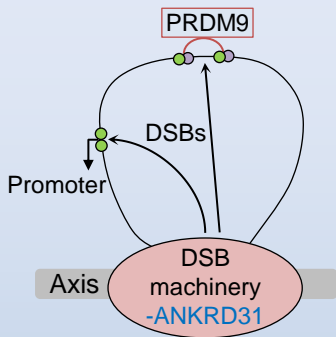
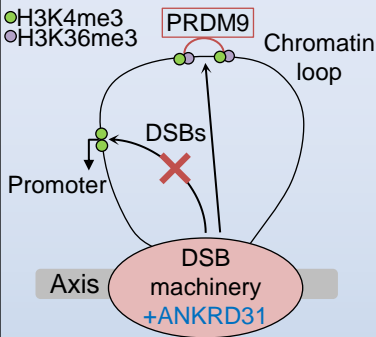




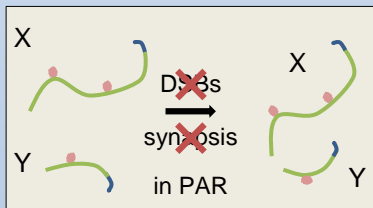
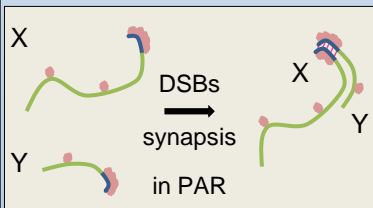
Wild-type

Ankrd31^{-/-}

Autosomes



Sex chromosomes



● DSB machinery - Axis - Synapsis - Pseudoautosomal region (PAR)