1 **Title:**

2 ***Trim41* is essential for preventing X chromosome**
 3 **chaotic synapsis in male mice.**

4
 5 **Running Title:**

6 *Trim41* is essential for male meiosis

7
 8 **Author Line:**

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49 Meiosis, Synapsis, XY body, Mouse, TRIM41

50

51 **Summary statement**

52 *Trim41*-disruption caused abnormal synapsis configuration of the X
53 chromosome and complete infertility in male mice. Thus, TRIM41 prevents the sex
54 chromosome from chaotic synapsis.

55 **Abstract**

56 Meiosis is a hallmark event in germ cell development that accompanies
57 sequential chromosome events executed by numerous molecules. Therefore,
58 characterization of these factors is one of the best strategies to clarify the mechanism of
59 meiosis. Here, we report tripartite motif-containing 41 (TRIM41), a ubiquitin ligase E3,
60 as an essential factor for proper meiotic progression and fertility in male mice. *Trim41*
61 KO spermatocytes exhibited synaptonemal complex protein 3 (SYCP3) overloading,
62 especially on the X chromosome, showing extensive self-synapsis of X chromosome
63 and non-homologous synapsis between the X chromosome and autosomes.
64 Furthermore, the mutant mice lacking the RING domain of TRIM41, required for the
65 ubiquitin ligase E3 activity, phenocopied *Trim41* KO mice. We then examined the
66 behavior of mutant TRIM41 (Δ RING-TRIM41) and found that Δ RING-TRIM41
67 accumulated on the chromosome axes with overloaded SYCP3. This result showed that
68 TRIM41 exerts the function on the chromosome axes. In summary, our study revealed
69 that *Trim41* is essential for preventing SYCP3 overloading and chaotic synapsis of the X
70 chromosome, suggesting a TRIM41-mediated mechanism for regulating unsynapsed
71 axes during male meiotic progression.

72

73 Main text

74 Introduction

75 Tripartite motif (TRIM) family proteins consist of more than 70 members in
 76 humans and mice (Ozato et al., 2008; Rajsbaum et al., 2014; Versteeg et al., 2013)
 77 (also according to NCBI and MGI databases). They contain three zinc-binding domains
 78 in the N-terminus: a RING finger domain, one or two B-Boxes (B1/B2) motifs, and a
 79 coiled-coil (CC) region (Fig 1A). The RING domains of many TRIM family members
 80 have been shown to confer E3 ubiquitin ligase activity (Hatakeyama, 2017; Rajsbaum et
 81 al., 2014) (Fig 1B) and implicated in various biological functions such as innate
 82 immunity and carcinogenesis regulation (Lassot et al., 2018; Zhang et al., 2013; Zhang
 83 et al., 2021). TRIM41-mediated ubiquitination also functions in natural immunity via
 84 target protein degradation, including B cell leukemia/lymphoma 10 (BCL10) (Yu et al.,
 85 2021), nucleoproteins of the influenza A virus, and vesicular stomatitis virus (Patil et al.,
 86 2020; Patil et al., 2018). Therefore, to examine the defects in natural immunity, we
 87 produced *Trim41* knockout (KO) mice. However, unexpectedly, *Trim41* KO male mice
 88 exhibited complete infertility due to meiotic defects. Thus, we focused on the analysis of
 89 meiotic events in this report.

90
 91 Here, meiosis is a hallmark event in germ cell development when homologous
 92 chromosomes undergo the physical juxtaposition (called synapsis) and shuffling of
 93 genetic material via a process known as homologous recombination. Upon entry into
 94 meiosis, in the leptotene stage, programmed DNA double-strand breaks (DSBs) are
 95 introduced (Keeney et al., 2014) and enable genome-wide search for homology through

96 the repair process, although a significant amount of chromosome pairing occurs before
97 the initiation of DSBs (Boateng et al., 2013). This homologous search drives the pairing
98 and alignment, finally leading to the synapsis of all homologs in the pachytene stage.
99 However, unlike autosomes that fully synapse between homologs, sex chromosomes (X
100 and Y in most mammals) have only synapsed at the pseudoautosomal region (PAR)
101 and sequestered into a physically separated nuclear territory known as the XY body.
102 The molecules involved in these complex chromosome events are not fully
103 characterized yet, especially in mammals, due to difficulties in culturing and genetically
104 manipulating spermatogenic cells *in vitro*. Thus, physiological screening with KO mice
105 has been a powerful strategy to identify meiosis-related factors (Gray and Cohen, 2016;
106 Oji et al., 2020; Oura et al., 2021).

107

108 In this study, we revealed that *Trim41* deficiency cause synaptonemal complex
109 protein 3 (SYCP3) overloading on several chromosome axes. Among them, the X
110 chromosome was mainly affected, exhibiting aberrant synapsis with autosomes rather
111 than forming the XY body. A transgene with a germ cell-specific expression rescued the
112 spermatogenesis defects, which showed that TRIM41 directly regulates meiosis.
113 Further, deletion of the RING domain (Δ RING) phenocopied *Trim41* KO. More
114 importantly, Δ RING-TRIM41 accumulated on chromosome axes with overloaded
115 SYCP3, suggesting that TRIM41-mediated protein degradation may act for removing
116 overloaded SYCP3. Thus, our study demonstrated that mammalian spermatocytes
117 have a TRIM41-mediated mechanism for preventing sex chromosome chaotic synapsis.

Results

***Trim41* is evolutionarily conserved between mammals and highly expressed in pachytene germ cells.**

The Treefam database (<http://www.treefam.org/>; Release 9, March 2013) shows that TF342569, a family containing TRIM41, has been annotated in 50% of eukaryotes, 98 % of vertebrates, and 100% of mammals (Fig. S1A). Also, phylogenetic analysis with Clustal W2.1(Larkin et al., 2007) showed that TRIM41 was highly conserved in many mammals, including cattle, dogs, mice, and humans (Figs 1C and S1B). Next, to determine the expression profile of *Trim41*, we performed RT-PCR using cDNA obtained from adult tissues, postnatal testis, and embryonic ovary. These RT-PCR showed that *Trim41* is expressed ubiquitously (Figs 1D–1F), albeit the most highly in testis (Fig. 1D). Furthermore, according to published single-cell RNA-sequencing (scRNA-seq) data analyzing mouse testis (Hermann et al., 2018) and embryonic ovary (Niu and Spradling, 2020), *Trim41* was the most highly expressed in pachytene germ cells (Figs S1C and S1D). These results suggest that TRIM41 functions during the meiotic phase of mammalian gametogenesis.

***Trim41* is essential for male fertility**

To uncover the function of *Trim41* *in vivo*, we generated *Trim41* KO mice by transfecting embryonic stem cells (ESCs) with a targeting vector (Fig. 1G; vector). Targeted ESC clones were injected into ICR embryos to obtain chimeric mice. The chimeric mice were mated with wild-type (WT) females to establish *Trim41* KO lines (Fig. 1H). KO mice obtained by heterozygous intercrosses showed no overt gross defects in development, behavior, and survival. Then, we housed individual *Trim41* KO male mice

142 with wild type (wt) females for two months to analyze their fertility. Although we
143 observed 19 vaginal plugs, *Trim41* KO males failed to sire pups (Fig. 1I). On the other
144 hand, we obtained pups from the mating pair of *Trim41* KO females and *Trim41* Het
145 males (7.4 ± 2.3 ; Fig. 1J), indicating that *Trim41* is dispensable for female fertility. As
146 *Trim41* Het male mice are fully fertile, we used littermate heterozygous males as
147 controls in some experiments.

148

149 **A transgene under *Clgn* promoter restored infertility of *Trim41* KO males**

150 To confirm infertility phenotype of *Trim41* KO males was due to *Trim41*
151 deficiency, not an aberrant genetic modification near the *Trim41* locus, and rule out the
152 possibility that latent systemic abnormalities affected male fertility, we carried out a
153 tissue-specific rescue experiment by generating transgenic (Tg) mouse lines. First, we
154 injected a DNA construct having PA-1D4-tagged *Trim41* under the testis-specific *Clgn*
155 promoter (Watanabe et al., 1995) (Fig. 1G; Tg) and established a Tg line (Fig. 1H;
156 Fw2-Rv3). *Clgn* promoter-driven *Trim41* expression started around postnatal day (PND)
157 9–11 (Fig. S2A), corresponding to the spermatocyte appearance. The Tg expression
158 was also confirmed by immunoblotting analysis using anti-PA (Fig. S2B) and -TRIM41
159 antibody (Fig. S2C), although the expression level of Tg was lower than that of intrinsic
160 *Trim41* (Fig S2C; WT v.s. KO-Tg). Then we housed *Trim41* KO male mice expressing
161 the Tg (referred to as KO-Tg) with WT females and observed the normal count of pups
162 (Fig. 1I), showing the *Clgn* promoter-driven *Trim41* Tg rescued the fertility of KO males.
163 These results indicated that *Trim41* expression from meiotic entry onward in testis is
164 essential for male fertility.

165

166 ***Trim41* KO male mice exhibited oligozoospermia**

167 When we observed gross testis morphology, *Trim41* KO testes were smaller
168 than those of control (testis/ body weight: $3.2 \pm 0.3 \times 10^{-3}$ [WT], $3.2 \pm 0.2 \times 10^{-3}$
169 [Het], $1.2 \pm 0.2 \times 10^{-3}$ [KO]; Figs 2A and 2B), indicating defective spermatogenesis in
170 *Trim41* KO testis. To define the cause of testicular atrophy, we performed hematoxylin
171 and periodic acid-Schiff (HePAS) staining of testicular sections. While three germ cell
172 layers existed in control testis sections, only two layers of germ cells existed in *Trim41*
173 KO testis (Fig. 2C; low magnification). The number of spermatocytes and spermatids
174 dramatically decreased in the KO testis (Fig. 2C; stage VII–VIII and XII). Although a few
175 elongating/elongated spermatids existed in the KO testis, their nuclei were not fully
176 compacted (Fig. 2C; red arrows). Consistent with the dramatic decrease of
177 spermatocytes and spermatids, the number of TUNEL positive cells increased in KO
178 testis compared with Het counterparts (Figs 2D and 2E). The TUNEL positive cells were
179 frequently observed in the second layer (Fig. 2D; high magnification). We did not see
180 the accumulation of TUNEL positive cells in a specific stage of the seminiferous
181 epithelium cycle (Fig. 2E). These results showed that spermatocytes after the
182 pachytene stage were gradually eliminated by apoptosis. As a result, only a few mature
183 spermatozoa existed in the cauda epididymis (Fig. 2F). In addition, all the spermatozoa
184 exhibited abnormal head/tail shapes (Fig. 2G) and were immotile. These *Trim41* KO
185 spermatogenesis defects were restored by *Clgn-Trim41* (Figs 2A–2F), albeit partially in
186 TUNEL analysis (Fig 2F). These observations suggested that *Trim41* KO males
187 exhibited spermatogenesis defects, leading to oligozoospermia.

188

189 ***Trim41* KO spermatocytes underwent SYCP3 overloading**

190 Due to an apparent defect during meiosis, we examined DNA DSBs and
191 synapsis by immunostaining surface chromosome spreads with anti- γ H2AX and SYCP3,
192 respectively (Figs 3A and 3B). The SYCP3/ γ H2AX immunostaining pattern in leptotene
193 and zygotene spermatocytes was comparable between the two genotypes, showing
194 that *Trim41* KO spermatocytes underwent programmed DSBs and initial assembly of
195 the synaptonemal complex. However, the compaction of the XY axes, a telltale
196 signature of XY body formation, was rarely observed in KO spermatocytes (Figs 3B and
197 3C). Consistent with the XY body malformation, the pachytene and diplotene
198 populations decreased dramatically in KO testis (Fig. 3D). More strikingly, intense
199 SYCP3 signals were observed on both autosomes and sex chromosomes (Figs 3B and
200 3C). The autosomes with the overloaded-SCYP3 tended to be inside of γ H2AX signals,
201 suggesting that the overloading of SCYP3 correlates with autosome unsynapsis.
202 Furthermore, we also observed multilayer SCYP3 axes in the X chromosome (Fig. 3C;
203 yellow arrowhead) and juxtaposition of sex chromosome and autosomes (Fig. 3C; red
204 arrowhead). Such overloading of SYCP3 became evident once homolog synapsis had
205 completed at pachytene rather than at zygotene/pachytene transition
206 (zygotene/pachytene transition; Figs S3A and S3B).

207 Since localization of axial element proteins, SYCP2 and SYCP3, depends on
208 cohesin axial core that is generated by REC8 and RAD21L (Fujiwara et al., 2020;
209 Ishiguro et al., 2014; Ishiguro, 2019), we examined the REC8 and RAD21L in *Trim41*
210 KO spermatocytes. Consistent with SYCP3 overloading, REC8 and RAD21L tended to
211 be hyperaccumulated (Fig. S4). Although RAD21L gradually dissociates from axial
212 elements from late pachytene onwards in WT spermatocytes (Fig. S4C) (Ishiguro et al.,
213 2011), the dissociation was evident in early pachytene of KO spermatocytes (Fig. S4D).

214 This observation is at least in part consistent with the evidence that Trim41 is
215 co-immunoprecipitated by RAD21L (Fujiwara et al., 2020).

216 Interestingly, the overloaded SYCP3 remained on a few chromosomes even in
217 metaphase I and anaphase I (Fig .3E; blue arrowheads). Further polycomplex-like
218 structure, aggregate of SYCP3, was also observed apart from chromosomes (Fig .3E;
219 magenta arrowheads). In addition, some chromosomes connected with the SYCP3
220 failed to align in the equatorial plate (Fig. 3E; Metaphase I) and were left behind
221 between two centrosomes (Fig. 3E; Anaphase I). Probably because of the remained
222 SYCP3 axes, one or two chromosomes failed to form bivalents and remained as
223 univalents at metaphase I (Fig. 3F). Because chromosome alignment requires a tension
224 across homologs during metaphase I (Kim et al., 2015; Woods et al., 1999), it was likely
225 that lack of chiasmata caused misalignment of those univalents. These results showed
226 that the SYCP3 amount on chromosome axes and synapsis configuration was
227 misregulated in *Trim41* KO spermatocytes.

228

229 **SYCP3 overloading were biased to the X chromosome**

230 Then, to examine whether SYCP3 overloading is biased to specific
231 chromosomes, we combined SYCP3 immunostaining with multicolor fluorescence in
232 situ hybridization (mFISH; Fig. 3G). The X chromosomes showed more frequent SYCP3
233 overloading than autosomes (Fig. 3H; X chromosome: 37 out 43 cells; autosome: 12 out
234 43 cells). Of note, the autosomes with overloaded SYCP3 signals were almost random
235 (Fig. 3H). Finally, we examined the pachytene stage in the E15.5 female ovary. The
236 gross gonad morphology was comparable between the two genotypes (Fig. S5A). The
237 SYCP3 overloaded axes were observed only in a few germ cells (Fig. S5B). This result

238 was contrast to the observation that SYCP3 overloading was frequent on X
239 chromosome in spermatocytes (Fig. 3H). As a result, folliculogenesis progressed
240 normally (Fig. S5C), and *Trim41* KO female mice were fully fertile (Fig. 1M). Overall,
241 these results showed that *Trim41* is essential for the proper behavior of the unsynapsed
242 X chromosome.

243

244 **SYCP3 overloaded X chromosome saw self- and non-homologous- synapsis.**

245 Next, to examine the synapsis state, we visualized the central element of the
246 synaptonemal complex (synapsed axes; Figs 4A and 4B) and unsynapsed axes (Figs
247 4C and 4D) by immunostaining surface chromosome spreads with anti- SYCP1 and
248 BRCA1 antibodies, respectively. SYCP1 is a molecule known to localize between the
249 lateral element of the synaptonemal complex of homologous chromosomes and form
250 transverse filaments, rungs of a ladder of synapsis (Liu et al., 1996; Meuwissen et al.,
251 1992). BRCA1, the product of the breast cancer 1, accumulates on unsynapsed axes of
252 sex chromosomes (Scully et al., 1997). Consistent with Fig. 3C (indicated by a red
253 arrow), X chromosome and autosome were connected by SYCP1 (Fig. 4B; white arrow)
254 in some spermatocytes. SYCP1 signals tended to remain on the overloaded SYCP3 of
255 X chromosomes (Fig. 4B), indicating that the X chromosomes synapse by themselves
256 or between sister chromatids. Strikingly, we observed multilayered SCYP1 signals in
257 some spermatocytes (Fig. 4B; blue arrows).

258 On the other hand, the BRCA1 staining pattern became uneven throughout the
259 X chromosomes (Fig. 4D; yellow and red arrows), indicating that the X chromosomes
260 have synapsed somehow. Furthermore, BRCA1 was positive on several autosomes
261 with overloaded SYCP3, suggesting that meiotic silencing of unsynapsed chromatin

(MUSC) was activated presumably as a result of delayed homolog synapsis at the SYCP3-overladed region. (Fig. 4D; green arrows). More notably, we observed the BRCA1 negative interface between the X chromosome and autosome axes (Fig. 4D; red arrow), suggesting that the X chromosome synapses with the autosome. These results indicated the X chromosome synapsed by themselves or with autosomes in *Trim41* KO spermatocytes.

268

***Trim41*^{ΔRING/ΔRING} male mice phenocopied *Trim41*^{neo/neo} male mice.**

To examine whether the RING domain is essential for *Trim41* function, we disrupted the RING domain by inserting the HA affinity tag sequence (Fig. 5A; referred to as ΔRING). The ΔRING-TRIM41 loses only ubiquitin ligase activity, not interaction with target proteins (Patil et al., 2020; Patil et al., 2018). We microinjected two gRNA/Cas9 ribonucleoprotein complexes and a ssODN into zygotes (Fig. 5A). Of 145 injected eggs, 119 eggs reached the two-cell stage. Then we transplanted the two-cell eggs into the oviducts of six pseudopregnant female mice and obtained twenty-five pups. The genotype PCR screening (Fig. 5B) detected the intended mutation with a 180 bp deletion and HA tag insertion (referred to as *Trim41*^{ΔRING}) from ten pups.

Immunoblotting analysis with anti-HA and -TRIM41 antibodies detected HA-tagged ΔRING-TRIM41 expression (Figs 5C and 5D). Then, we caged *Trim41*^{ΔRING/ΔRING} (referred to as ΔR-Homo; *Trim41*^{wt/ΔRING}: referred to ΔR-Het) male mice with WT females for two months to analyze their fertility. Although we confirmed 38 vaginal plugs, *Trim41* ΔR-Homo males failed to sire any pups (Fig. 5E) as well as *Trim41* KO males. Further, testis of *Trim41* ΔR-Homo males was smaller than the ΔR-Het counterparts (Figs 5F and 5G) due to spermatogenesis defects (Fig. 5H). The TUNEL-positive cells were

286 frequently observed in the 2nd layer of tubules (Fig. 5I). Also, TUNEL signals were
287 positive in overall seminiferous epithelial cycles (Fig. 5J). As a result, almost no
288 fully-matured spermatids existed in the cauda epididymis of ΔR -Homo males (Fig. 5K).
289 These results showed that *Trim41* ^{$\Delta RING/\Delta RING$} male mice exhibited the same phenotype
290 as *Trim41*^{neo/neo} male mice, suggesting that the RING domain is essential for TRIM41
291 function.

292

293 **$\Delta RING$ -TRIM41 accumulated on SYCP3-overloaded region**

294 Immunostaining analysis with anti-SYCP3 and γ H2AX antibodies
295 demonstrated XY body malformation and SYCP3 overloading in ΔR -Homo pachytene
296 spermatocytes (Figs 6A and 6B). Also, the number of pachytene and diplotene
297 spermatocytes decreased dramatically in ΔR -Homo testis (Figs S6A–C). These results
298 corroborated the necessity of the RING domain in TRIM41 functions.

299 Next, to determine TRIM41 localization, we stained the spread nuclei with an
300 anti-HA antibody (Figs 6C–F). In ΔR -Het spermatocytes, we observed only faint signals
301 throughout the nuclei (Fig. 6C). However, surprisingly, ΔR -Homo spermatocytes saw
302 intense HA signals on the SYCP3-overloaded regions (Figs 6D and S6A; white arrows).
303 The strong HA signals appeared after the zygotene–pachytene transition (Figs S7A). In
304 addition, the HA signals tended to become stronger during prophase I progression (Figs
305 S7A), showing a correlation with SYCP3 overloading. The HA foci were also detected in
306 *Trim41*^{neo/ $\Delta RING$} spermatocytes (Fig. 6E), demonstrating that the removal of the RING
307 domain changed the immunostaining pattern, not the protein level difference. On the
308 other hand, we did not detect the HA immunostaining signals in *Trim41* KO
309 spermatocyte with SYCP3 overloading (Fig. 6F), ruling out the possibility of antibody

310 cross-reaction. Furthermore, we confirmed the same phenomena in immunostaining
311 analysis with an anti-TRIM41 antibody (Figs S7B–E). These observations showed that
312 Δ RING-TRIM41 accumulated on the SYCP3-overloaded regions, suggesting that
313 TRIM41 functions on the chromosome axes. As a side note, we also identified several
314 HA foci outside of the chromosome axes in zygotene and early pachytene
315 spermatocytes (Figs 6D and S7A; blue arrows), although we could not specify the
316 location of those foci.

317

Δ RING-TRIM41 accumulated on the synapsed part of the X chromosome.

319 Finally, we examined the relationship between Δ RING-TRIM41 accumulation
320 and the synapsis state by SYCP1 and BRCA1 immunostaining. First, we confirmed
321 SYCP1 tended to remain on the sex chromosome axes in Δ R-Homo spermatocytes and
322 KO spermatocytes, although the sex chromosome SYCP1 axes were uneven (Figs. 6G
323 and S8A). Then, we compared the SYCP1 immunostaining pattern with the
324 Δ RING-TRIM41 accumulation pattern and noticed that the boundary of the SYCP1
325 positive and negative parts were hotspots of Δ RING-TRIM41 accumulation (Figs. 6G
326 and S8A; magenta arrowheads). More strikingly, the Δ RING-TRIM41 also existed on the
327 interface between the X chromosome and autosomes (Figs. 6G and S8A; yellow
328 arrowheads). On the other hand, in BRCA1/HA immunostaining analysis, we observed
329 Δ RING-TRIM41 on the BRCA1 negative part of the X chromosome axes (Figs. 6H and
330 S8B; yellow arrowheads). Above all, these observations suggested that Δ RING-TRIM41
331 accumulated on the synapsed part of the X chromosome axes, and therefore TRIM41 is
332 essential for preventing the chaotic X chromosome synapsis.

333 Given that TRIM41 confers ubiquitin ligase E3 activity (Patil et al., 2020; Patil et
334 al., 2018), we tried to identify the substrates by immunoprecipitation. However, we could
335 not detect SYCP3 in co-IPed elute samples by immunoblotting (Figs S9A–S9F). Also,
336 we could not find candidate proteins in IP-Mass analysis (Figs S9G–S9I). Thus,
337 substrate discovery would be the feature research.

338 Discussion

339 In this research, we produced *Trim41* KO mice and analyzed the phenotype.
 340 Unexpectedly, *Trim41* deficiency caused meiosis defects and infertility in male mice.
 341 The infertility was rescued by a transgene driven by a testis-specific *Clgn* promoter. In
 342 detailed analyses, we found that *Trim41* KO spermatocytes exhibited SYCP3
 343 overloading. We also noticed that and the X chromosomes underwent self- and
 344 non-homologous- synapsis. Furthermore, we removed the RING domain of TRIM41
 345 (Δ RING) to examine the necessity of the RING domain and found that *Trim41* ^{Δ RING/ Δ RING}
 346 phenocopied *Trim41*^{neo/neo} (i.e., KO). Surprisingly, the Δ RING-TRIM41 accumulated on
 347 the SYCP3-overloaded regions. The Δ RING-TRIM41 accumulation also correlated with
 348 the chaotic X chromosome synapsis.

349
 350 The most striking phenotype of *Trim41* KO spermatocytes was SYCP3
 351 overloading. In addition, some X chromosomes exhibited multilayered SYCP3 signals,
 352 clearly demonstrating self-synapsis of the X chromosome. Although we could not
 353 specify, this self-synapsis might be inter-sister synaptonemal complex assembly like
 354 those found in REC8 KO spermatocytes (Ishiguro et al., 2014; Ishiguro and Watanabe,
 355 2016; Xu et al., 2005). However, given that we also observed multilayer SYCP1 signals
 356 in the X chromosome, we should also consider the possibility of extensive heterologous
 357 self-synapsis like those seen in domestic dog pachytene spermatocytes (Federici et al.,
 358 2015). The latter possibility is corroborated by another outstanding phenotype of the
 359 non-homologous synapsis between the X chromosome and autosomes. Further studies
 360 are needed to determine the relationship between these two phenotypes (i.e., SYCP3
 361 overloading and abnormal synapsis configuration; causal relationship or simultaneous

phenomena) and whether these phenotypes are direct influences of TRIM41 deficiency or compensatory changes.

To examine which chromosomes exhibited SYCP3 overloading in *Trim41* KO spermatocytes, we performed multicolor FISH combined with SYCP3 immunostaining and found that the X chromosome was mainly affected. On the other hand, autosomes saw fewer SYCP3 overloading and no biases to specific chromosomes. A possible reason for the X chromosome bias is the unsynapsed nature during the pachytene stage. This inference agrees that only a tiny population of germ cells underwent SYCP3 overloading in *Trim41* KO females, whose X chromosomes are fully synapsed during prophase I progression. Therefore, *Trim41*-deficient XO meiosis might be an intriguing future consideration.

To examine the molecular function of TRIM41, we also produced *Trim41*^{ΔRING/ΔRING} mice by replacing 2–61 aa residues with HA-tag sequence, and we found that the HA immunostaining signals on overloaded-SYCP3 region. As the HA signals appeared only when the cells had no functional TRIM41 (i.e. *Trim41*^{ΔRING/ΔRING} or *Trim41*^{neo/ΔRING}), we concluded that ΔRING-TRIM41 accumulated on the SYCP3-overloaded regions. This observation suggested that ΔRING-TRIM41 kept targeting the ubiquitinated substrates, and therefore TRIM41 exerts its ubiquitin ligase E3 activity on the chromosome axes. However, we could not narrow down the substrate candidate by IP-Mass analysis. Also, we did not see the interaction between TRIM41 and SYCP3. As the weak/transient binding between enzymes and substrates tends to be challenging to capture in IP analysis (Trinkle-Mulcahy, 2019), the recently

386 developed proximity-dependent biotin identification (BioID) technique (Kim et al., 2016;
387 Roux et al., 2012) could be a future consideration in substrate identification.

388

389 In summary, our result showed that *Trim41* is essential for preventing SYCP3
390 overloading and X chromosome chaotic synapsis. Further studies for substrate
391 identification will unveil the exact molecular functions of TRIM41 and the mechanism for
392 safeguarding X chromosome chaotic synapsis in male meiosis.

393 **Material & methods**

394 ***Animals***

395 All animal experiments were approved by the Animal Care and Use Committee
396 of the Research Institute for Microbial Diseases, Osaka University
397 (#Biken-AP-R03-01-0). Animals were housed in a temperature-controlled environment
398 with 12 h light cycles and free access to food and water. B6D2F1 (C57BL/6 × DBA2;
399 Japan SLC, Shizuoka, Japan) mice and ICR (SLC) were used as embryo donors;
400 B6D2F1 were used for mating and wild-type control; C57BL6/N (SLC) mice were used
401 to collect RNA for cloning.

402

403 ***Generation of Trim41 KO mice***

404 *Trim41* KO mice were generated by transfecting EGR-G101 (Fujihara et al.,
405 2013) with a targeting vector. Then, potentially targeted ES cell clones were selected
406 with neomycin. Correctly targeted ES cell clones and germ-line transmission were
407 confirmed via PCR using primers (GeneDesign, Osaka, Japan) listed in Table S1.
408 Finally, the heterozygous KO mice were mated with B6D2F1 and then maintained by
409 sibling mating. The B6D2 KO mouse line is available from the Riken BioResource
410 Center (Riken BRC, Tsukuba, Japan; #11261) and the Center for Animal Resources and
411 Development, Kumamoto University (CARD, Kumamoto, Japan; #3019).

412

413 ***Generation of Trim41 transgenic mice***

414 The mouse *Trim41* cDNA (ENSMUST00000047145) tagged with PA and 1D4
415 was inserted under the control of the mouse Cln promoter (Addgene #173686) (Ikawa
416 et al., 2001; Watanabe et al., 1995). The *Trim41* cDNA inserted plasmids are deposited

417 at Riken BRC and Addgene. After linearization, the DNA construct (2.16 ng/μL; 0.54
418 ng/μL/kbp) was injected into the pronucleus of fertilized eggs. The injected eggs were
419 transplanted into the oviduct ampulla of pseudopregnant mice (ICR; 10 embryos per
420 ampulla). After 19 days, pups were delivered through Cesarean section and placed
421 with foster mothers (ICR). For the rescue experiment, F0 Tg mice were mated with
422 *Trim41* KO mouse line. The mouse colony with the transgene and KO allele was
423 maintained by sibling mating. The genotyping primers (GeneDesign) are listed in Table
424 S1. The transgenic mouse line is available from Riken BRC (#11216) and CARD
425 (#3019).

426

427 ***Generation of Δ RING-Trim41 mice***

428 *Δ RING-Trim41* mice were generated by microinjection described previously
429 (Oura et al., 2020). First, a gRNA solution was prepared by annealing two tracrRNAs
430 (Sigma-Aldrich, St. Louis, MO, USA) and crRNA (Sigma-Aldrich). The target genomic
431 sequences are listed in Table S1. Then, the gRNA solution and Cas9 nuclease solution
432 (Thermo Fisher Scientific, Waltham, MA, USA) were mixed: 40 ng/μL gRNA each and
433 108 ng/μL Cas9 nucleases. The obtained complex was then microinjected into fertilized
434 eggs (B6D2F1) using a programmable microinjector (FemtoJet 4i, Eppendorf, Hamburg,
435 Germany). The microinjected eggs were then transplanted into the oviduct ampulla of
436 pseudopregnant mice (ICR) on the following day. After 19 days, pups were delivered
437 through Cesarean section and placed with foster mothers (ICR). To generate
438 heterozygous mutant mice, F0 mice were mated with WT B6D2F1. Mouse colonies with
439 the desired mutation were maintained by sibling mating. The genotyping primers
440 (GeneDesign) are listed in Table S1. The mutant mouse line is available from Riken

441 BRC (#11041) and CARD (#2948).

442

443 ***Bacterial strains***

444 *Escherichia coli* (*E. coli*) strain DH5 α (Toyobo, Osaka, Japan) and BL21(de3)
445 pLysS (C606003, ThermoFisher Scientific) were used for DNA cloning and protein
446 expression, respectively. *E. coli* cells were grown in LB or 2 $\times$ YT medium containing 100
447 mg/L ampicillin and were transformed or cloned using standard methods.

448

449 ***Production of anti-TRIM41 antibody***

450 Monoclonal antibody against TRIM41 was produced as previously described
451 (Oura et al., 2021). The DNA encoding mouse TRIM41 (residue 35-85 aa,
452 NP_663352.2) was inserted into pGEX6p-1 (GE healthcare), and the expression vector
453 was transformed into *E. coli* strain BL21 (de3) pLysS (C606003, Thermo Fisher
454 Scientific). GST-TRIM41 was purified using Glutathione Sepharose 4B (GE Healthcare).
455 The GST tag was removed by PreScission protease (27084301, GE Healthcare) and
456 Glutathione Sepharose 4B affinity subtraction purification. The purified TRIM41 protein
457 with a complete adjuvant was injected into female rats. After 17 days of injection,
458 lymphocytes were collected from iliac lymph nodes for hybridomas generation (Kishiro
459 et al., 1995; Sado et al., 2006). The hybridomas were cloned by a limited dilution and
460 screened by ELISA against recombinant TRIM41 and immunoblotting against testis
461 lysate. A monoclonal antibody from hybridoma clone #33-16 was used in this study. As a
462 side note, the #33-16 antibody also recognized Δ RING-TRIM41 missing 2-61 aa
463 residues, showing that the epitope of #33-16 is in 59-85 aa residues (two or three
464 mismatches might be acceptable).

465

466 ***Genotype analysis***

467 PCR was performed using KOD FX neo (KFX-201, TOYOBO). The primers
468 (GeneDesign) for each gene are summarized in Table S1. PCR products were purified
469 using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) kit
470 for Sanger sequencing.

471

472 ***Sequence comparison analysis***

473 Amino acid sequences of TRIM41 were obtained from the NCBI Entrez Protein
474 database. Clustal W2.1 was used for multiple sequence alignment (Larkin et al., 2007).

475

476 ***Immunoblotting***

477 Proteins from testis were extracted using NP40 lysis buffer [50mM Tris-HCl (pH
478 7.5), 150 mM NaCl, 0.5% NP-40, 10% Glycerol]. Proteins were separated by
479 SDS-PAGE under reducing conditions and transferred to polyvinylidene fluoride (PVDF)
480 membrane using the Trans Blot Turbo system (BioRad, Munich, Germany). After
481 blocking with 10% skim milk (232100, Becton Dickinson, Cockeysville, MD, USA), the
482 membrane was incubated with primary antibody overnight at 4°C, and then incubated
483 with HRP-conjugated secondary antibody for 1 h at room temperature.
484 Chemiluminescence was detected by ECL Prime Western Blotting Detection Reagents
485 (RPN2232, GE Healthcare, Chicago, IL, USA) using the Image Quant LAS 4000 mini
486 (GE Healthcare). The antibodies used in this study are listed in Table S2.

487

488 ***Fertility analysis***

489 To examine fertility, sexually mature male mice were caged with wild-type
490 females (B6DF1) for at least three months. The vaginal plugs and pup's numbers were
491 recorded at approximately 10 AM to determine the number of copulations and litter size.
492 Numerical data is available in Table S3.

493

494 **Morphological and histological analysis of testis and epididymis**

495 To observe gross testis morphology and measure testicular weight, mice over
496 11-week-old age were euthanized after measuring their body weight. Numerical data is
497 available in Table S3. The whole testis was observed using BX50 and SZX7 (Olympus,
498 Tokyo, Japan) microscopes. For histological analysis, testes were fixed in Bouin's
499 fixative solution (16045-1, Polysciences, Warrington, PA, USA) at 4°C O/N, dehydrated
500 in increasing ethanol concentrations and 100% xylene, embedded in paraffin, and
501 sectioned (5 µm). The paraffin sections were hydrated with 100% Xylene and
502 decreasing ethanol concentrations, treated with 1% periodic acid (26605-32, Nacalai
503 Tesque, Kyoto, Japan) for 10 min, incubated with Schiff's reagent (193-08445, Wako)
504 for 20 min, counterstained with Mayer's hematoxylin solution (131-09665, Wako) for 3
505 min, dehydrated in increasing ethanol concentrations, and finally mounted with
506 Permount (SP15-100-1, Ferma, Tokyo, Japan). The sections were observed using a
507 BX53 (Olympus) microscope. Seminiferous tubule stages were identified based on the
508 morphological characteristics of the germ cell nuclei (Ahmed and de Rooij, 2009).

509

510 ***Apoptosis detection in testicular section***

511 TdT-mediated dUTP nick end labeling (TUNEL) staining was carried out with In
512 Situ Apoptosis Detection Kit (MK500, Takara Bio Inc., Shiga, Japan), according to the

513 manufacturer's instruction. Briefly, testes were fixed with Bouin's fixative, embedded in
514 paraffin, and sectioned (5 μ m). After paraffin removal, the slides were boiled in citrate
515 buffer (pH 6.0; 1:100; ab93678, abcam, Cambridge, UK) for 10 min and incubated in 3%
516 H₂O₂ at room temperature for 5 min for endogenous peroxidase inactivation, followed by
517 a labeling reaction with TdT enzyme and FITC-conjugated dUTP at 37°C for 1 h.

518 For chromogenic detection of apoptosis, the sections were incubated with
519 HRP-conjugated anti-FITC antibody at 37°C for 30 min. The section was then incubated
520 in ImmPACT DAB (SK-4105, Vector Laboratories, Burlingame, CA, USA) working
521 solution, counterstained with Mayer's hematoxylin solution for 3 min, dehydrated in
522 increasing ethanol concentrations, and finally mounted with Permount. The sections
523 were observed using a BX53 (Olympus) microscope. Seminiferous tubule stages were
524 identified based on the morphological characteristics of the germ cell nuclei (Ahmed and
525 de Rooij, 2009). Numerical data is available in Table S3.

526

527 ***Immunostaining of surface chromosome spread***

528 Spread nuclei from spermatocytes were prepared as previously described (Oji
529 et al., 2020; Oura et al., 2021). Seminiferous tubules were unraveled using forceps in
530 ice-cold DMEM (11995065, Thermo Fisher Scientific) and incubated in 1 mg/mL
531 collagenase (C5138, Sigma-Aldrich) in DMEM (20 mL) at 37°C for 15 min. After three
532 washes with DMEM, the tubules were transferred to 20 mL trypsin/DNaseI medium
533 [0.025 w/v% trypsin, 0.01 w/v% EDTA, 10U DNase in DMEM] and incubated at 37 °C for
534 10 min. After adding 5 mL of heat-inactivated FCS and pipetting, the solution was
535 filtered through a mesh (59 μ m; N-N0270T, NBC Meshtec inc., Tokyo, Japan) to remove
536 tubule debris. The collected testicular cells were resuspended in hypotonic solution [100

537 mM sucrose] and 10µL of the suspension was dropped onto a slide glass with 100 µL of
538 fixative solution [1% PFA, 0.1% (v/v) Triton X-100]. The slides were then air-dried and
539 washed with PBS containing 0.4% Photo-Flo 200 (1464510, Kodak Alaris, NY, USA) or
540 frozen for longer storage at -80°C.

541 The spread samples were blocked with 10% goat serum in PBS and then
542 incubated with primary antibodies overnight at 4°C in blocking solution. After incubation
543 with AlexaFlour 488/546-conjugated secondary antibody (1:200) at room temperature
544 for 1 h, samples are counterstained with Hoechst 33342 and mounted with Immu-Mount.
545 The samples were observed using a BX53 (Olympus) microscope. The antibodies used
546 in this study are listed in Table S2. Numerical data is available in Table S3.

547

548 **Giemsa staining of metaphase I chromosome spreads**

549 For preparing metaphase chromosome spreads, seminiferous tubules were
550 unraveled using forceps in ice-cold PBS and transferred to a 1.5-mL tube with 1 mL of
551 accutase (12679–54, Nacalai Tesque), followed by clipping the tubules, and a 5 min
552 incubation at room temperature. After filtration with a mesh (59 µm; N-N0270T, NBC
553 Meshtec inc.) and centrifugation, the cells were resuspended in 8 mL of hypotonic
554 solution [1% sodium citrate] and incubated for 5 min at room temperature. The
555 suspension was centrifuged and 7 mL of supernatant was aspirated. The cells were
556 then resuspended in the remaining 1 mL of supernatant and 7 mL of Carnoy's Fixative
557 [75% Methanol, 25% Acetic Acid] were added gradually while shaking. After 2 washes
558 with Carnoy's Fixative, the cells were resuspended in ~ 0.5 mL of Carnoy's Fixative and
559 dropped onto a wet glass slide. The slide was stained with Giemsa Stain Solution
560 (079–04391, wako) and observed using a BX53 (Olympus) microscope.

561

562 ***Multicolor fluorescence in situ hybridization (mFISH) followed by SYCP3***
 563 ***immunostaining***

564 Testicular germ cells were suspended in 3 mL of hypotonic solution [0.075%
 565 potassium chrolide] and incubated for 20 min at room temperature. Then the cells were
 566 mixed with 1 mL of Carnoy's Fixative [75% Methanol, 25% Acetic Acid] to the
 567 suspension for fixing. After 5 mL of Carnoy's Fixative addition, the suspension was
 568 again mixed and centrifuged at 1,500 rpm for 5 minutes. After three washes with 5 mL of
 569 Carnoy's Fixative, the fixed cells were subjected to metaphase spreader Hanabi
 570 (ADSTEC, Funabashi, Japan) for spread sample preparation.

571 For multicolor FISH analysis, the spread samples were hybridized with
 572 21XMouse (MetaSystems, Altlussheim, Germany) according to the manufacturer's
 573 protocol. For denaturation of the nuclear DNA, the spread samples were incubated in
 574 2×SSC for 30 min at 70°C and then treated with 0.07M NaOH for 1 min at room
 575 temperature. The denatured samples were washed with 0.1×SSC and 2×SSC for 1 min
 576 at 4°C, respectively, and then dehydrated with 70%, 95%, and 100% ethanol. Multicolor
 577 FISH probes were denatured for 5 min at 75°C and applied to the spread samples. After
 578 hybridization for 48 h at 37°C in a humidified chamber, the spread samples were treated
 579 with 0.4× SSC for 2 min at 72°C, washed in 2×SSC containing 0.05% Tween20 for 30
 580 sec at room temperature, and rinsed with distilled water.

581 Immunostaining of the spread nuclei with SYCP3 was performed described
 582 above. After incubation of the spread samples with primary and secondary antibodies,
 583 the slides were covered by a coverslip with DAPI/Antifade (MetaSystemes, Altlussheim,
 584 Germany) and observed under a fluorescent microscope. Fluorescence images were

585 captured using a high-sensitive digital camera (α 7s, SONY, Tokyo, Japan), and the
586 chromosome numbering of each synaptonemal complex was determined based on the
587 fluorescence color.

588

589 ***Immunoprecipitation***

590 Proteins from seminiferous tubules were extracted using NP40 lysis buffer [50
591 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.5% NP-40, 10% Glycerol]. Protein lysates were
592 mixed with 20 μ L Protein G-conjugated magnetic beads (DB10009, Thermo Fisher
593 Scientific) with 2.0 μ g antibody. The immune complexes were incubated for 1 h at 4°C
594 and washed 3 times with NP40 lysis buffer. Co-immunoprecipitated products were then
595 eluted by resuspension in 2x SDS sample buffer [125 mM Tris-HCl (pH6.8), 10%
596 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 10% sucrose, 0.01%
597 bromophenol blue] and 10 min incubation at 70°C. The antibodies used in this study are
598 listed in Table S2.

599

600 ***Mass spectrometry and data analysis.***

601 Before MS analysis, half of the eluted amount was subjected to SDS-PAGE
602 and silver staining (06865-81, Nacalai Tesque). The remaining half amount was
603 subjected to mass spectrometry (MS) analysis. The proteins were reduced with 10 mM
604 dithiothreitol (DTT), followed by alkylation with 55 mM iodoacetamide, and digested by
605 treatment with trypsin and purified with a C18 tip (GL-Science, Tokyo, Japan). The
606 resultant peptides were subjected to nanocapillary reversed-phase LC-MS/MS analysis
607 using a C18 column (25 cm $\times$ 75 μ m, 1.6 μ m; IonOpticks, Victoria, Australia) on a
608 nanoLC system (Bruker Daltoniks, Bremen, Germany) connected to a tims TOF Pro

609 mass spectrometer (Bruker Daltoniks) and a modified nano-electrospray ion source
 610 (CaptiveSpray; Bruker Daltoniks). The mobile phase consisted of water containing 0.1%
 611 formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Linear
 612 gradient elution was carried out from 2% to 35% solvent B for 18 min at a flow rate of
 613 400 nL/min. The ion spray voltage was set at 1.6 kV in the positive ion mode. Ions were
 614 collected in the trapped ion mobility spectrometry (TIMS) device over 100 ms and MS
 615 and MS/MS data were acquired over an m/z range of 100-1,700. During the collection of
 616 MS/MS data, the TIMS cycle was adjusted to 1.1 s and included 1 MS plus 10 parallel
 617 accumulation serial fragmentation (PASEF)-MS/MS scans, each containing on average
 618 12 MS/MS spectra (>100 Hz), and nitrogen gas was used as the collision gas.

619 The resulting data were processed using DataAnalysis version 5.1 (Bruker
 620 Daltoniks), and proteins were identified using MASCOT version 2.6.2 (Matrix Science,
 621 London, UK) against the SwissProt database. Quantitative value (available in Table S4)
 622 and fold exchange were calculated by Scaffold4 (Proteome Software, Portland, OR,
 623 USA) for MS/MS-based proteomic studies.

624

625 ***Statistics and Reproducibility***

626 All error bars indicated standard deviation. The sample numbers were
 627 described in each legend or/and in the figure panel.

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634

635 **Competing interests**

636 The authors declare no competing interests.

637

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645

646 **Data availability**

647 The authors declare that the data supporting this study's findings are available
648 from the corresponding author upon request. Numerical data that underlies graphs were
649 listed in Table S3.

650

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774

775 **Figure legends**

776 **Fig. 1: Production of *Trim41* KO mice and fertility analysis.**

777 (A) Schematic of TRIM41 protein structure and antigen position. (B) Model of TRIM E3
778 ubiquitin ligase activity. The RING domain at the N terminus interacts with E2
779 ubiquitin-conjugating enzyme. The C terminal part of TRIM recognizes the substrates.
780 (C) Phylogenetic tree constructed by ClustalW with TRIM41 sequences of various
781 mammals. (D) RT-PCR using multi-tissue cDNA. *Actb* was used as a loading control.
782 (E) RT-PCR using postnatal testis cDNA. (F) RT-PCR using embryonic ovary cDNA. (G)
783 Targeting scheme for *Trim41* disruption and a Tg construct. Black and white boxes in the
784 gene map indicate coding and non-coding regions, respectively. Black arrowheads
785 (Primer F1, F2, R1, R2, and R3) indicate primers for genotyping. (H) An example of
786 genotyping PCR with primer sets in G. (I) The result of mating tests. Pups/plugin: 8.9±1.7
787 [Het]; 0 [KO]; 9.4±2 [KO-Tg] (s.d.). Error bars indicate standard deviation. The numerical
788 data is available in Table S3. (J) Pup numbers from mating pairs of *Trim41* KO females
789 and *Trim41* Het males (7.4±2.3; s.d.). Error bars indicate standard deviation. The
790 numerical data is available in Table S3.

791

792 **Fig. 2: Histological analysis of *Trim41* KO male mice.**

793 (A and B) Testis morphology (A) and testis/bodyweight of WT, *Trim41* Het, *Trim41* KO,
794 and KO-Tg adult mice (B). Testis/body weight: 3.2±0.3 x 10⁻³ [WT], 3.1±0.2 x 10⁻³
795 [Het] , 1.2±0.2 x 10⁻³ [KO], 2.8±0.1 x 10⁻³ [KO-Tg]. Error bars indicate standard
796 deviation. The numerical data is available in Table S3. (C) PAS staining of seminiferous
797 tubules of adult mice. The seminiferous epithelium cycle was determined by germ cell
798 position and nuclear morphology. Red arrows indicate elongated spermatids with

799 abnormal head morphology. At least three male mice were analyzed. (D) TUNEL
800 staining of seminiferous tubules of adult mice counterstained with hematoxylin. At least
801 three male mice were analyzed. (E) The stacked bar graph of TUNEL positive cells. The
802 seminiferous epithelial stages were roughly determined by the arrangement and nuclear
803 morphology of the first layer of germ cells (spermatogonia and leptotene/zygotene
804 spermatocytes). The numerical data is available in Table S3. (F) PAS staining of cauda
805 epididymis of adult mice. At least three male mice were analyzed. (G) Spermatozoa
806 extracted from cauda epididymis of adult mice.

807

808 **Fig. 3: Cytological analysis of *Trim41* KO spermatocytes.**

809 (A and B) Immunostaining of spread nuclei from prophase spermatocytes collected from
810 adult Het (A) and KO (B) male mice. White arrows indicate overloaded SYCP3 axes. At
811 least three male mice were analyzed. (C) Additional immunostaining images of KO
812 spermatocytes. The yellow arrowhead indicates the X chromosome with two SYCP3
813 axes. The red arrowhead shows the evident synapsis between the sex chromosome
814 and autosomes (only 17 axes existed outside the γ H2AX cloud). (D) The percentage of
815 each meiotic prophase stage in immunostained spread nuclei samples in A and B. The
816 numerical data is available in Table S3. (E) Immunostaining of metaphase and
817 anaphase spermatocytes squashed from seminiferous tubules after fixation. Magenta
818 and blue arrowheads indicate polycomplex-like structures, aggregate of SYCP3, and
819 chromosomes connected with the polycomplex-like structures. At least three male mice
820 were analyzed. (F) Giemsa staining of spread nuclei of metaphase I spermatocytes.
821 Black arrows indicate the miscondensation of chromosomes. At least three male mice
822 were analyzed. (G) Multicolor FISH followed by SYCP3 immunostaining. White arrows

823 indicate overloaded SYCP3 axes. (H) The histogram for the chromosome distribution of
824 SYCP3 overloaded axes. Forty-three well-spread cells were analyzed.

825

826 **Fig. 4: Synapsis configuration of SYCP3 overloaded axes.**

827 (A and B) SYCP1 immunostaining of spread nuclei from prophase spermatocytes
828 collected from adult Het (A) and KO (B) male mice. The sex chromosomes were
829 identified by their characteristic shape of SYCP3 axes. At least three male mice were
830 analyzed. (C and D) BRCA1 immunostaining of spread nuclei from prophase
831 spermatocytes collected from adult Het (C) and KO (D) male mice. At least three male
832 mice were analyzed.

833

834 **Fig. 5: Production and phenotypical analysis of *Trim41*^{ΔRING/ΔRING} mice.**

835 (A) Knock-in scheme for the replacement of the RING domain with HA tag. Black and
836 white boxes in the gene map indicate coding and non-coding regions, respectively.
837 Black arrowheads (F3 and R4) and arrows (gRNA1 and gRNA2) indicate primers for
838 genotyping and target sequence of gRNAs, respectively. (B) An example of genotyping
839 PCR with primer sets in A. (C and D) Immunoblotting analysis with anti-HA (C) and
840 -TRIM41 (D) antibodies. Black and red arrows indicate TRIM41 and HA-ΔRING-TRIM41,
841 respectively. (E) The result of mating tests. Pups/plug: 9.1±2.5 [WT]; 0 [ΔR-Homo] (s.d.).
842 Error bars indicate standard deviation. The numerical data is available in Table S3. (F
843 and G) Testis morphology (F) and testis/bodyweight of ΔR-Het and ΔR-Homo adult mice
844 (G). Testis/body weight: 3.6±0.0 x 10⁻³ [ΔR-Het]; 1.5±0.2 x 10⁻³ [ΔR-Homo]. Error bars
845 indicate standard deviation. The numerical data is available in Table S3. (H) PAS
846 staining of seminiferous tubules of adult mice. The seminiferous epithelium cycle was

determined by germ cell position and nuclear morphology. At least three male mice were analyzed. (I) TUNEL staining of seminiferous tubules of adult mice counterstained with hematoxylin. At least three male mice were analyzed. (J) The stacked bar graph of TUNEL positive cells. The seminiferous epithelial stages were roughly determined by the arrangement and nuclear morphology of the first layer of germ cells (spermatogonia and leptotene/zygotene spermatocytes). The numerical data is available in Table S3. (K) PAS staining of cauda epididymis of adult mice. At least three male mice were analyzed.

855

Fig. 6: Cytological analysis of *Trim41*^{ΔRINGΔRING} spermatocytes.

(A and B) SYCP3/γH2AX immunostaining of pachytene spermatocytes from ΔR-Het (A) and ΔR-Homo (B) male mice. At least three male mice were analyzed. (C–F) SYCP3/HA immunostaining of pachytene spermatocytes from ΔR-Het (C), ΔR-Homo (D), *Trim41*^{neo/ΔRING} (E), and KO (F) male mice. White and blue arrowheads indicate HA signals on and outside of SYCP3 axes, respectively. At least three male mice were analyzed for ΔR-Het and ΔR-Homo. (G) SYCP1/HA immunostaining of surface chromosome spread from ΔR-Homo testis. Magenta and yellow arrowheads indicate HA signals on the SYCP1 positive/negative boundary and the non-homologous axis interface, respectively. At least three male mice were analyzed. (H) BRCA1/HA immunostaining of surface chromosome spread from ΔR-Homo testis. Red arrowheads indicate HA signals on the BRCA1 negative part of the X chromosome axes. At least three male mice were analyzed.

Supplemental information

Fig. S1: *Trim41* is an evolutionally conserved gene expressed highly in pachytene germ cells.

(A) The percentage of TF342569 annotated species based on TreeFam database (Release 9; <http://www.treefam.org/>). Dark and light green show species with and without TF342569 annotation, respectively. (B) Protein sequence comparison of TRIM41 in big brown bat (XP_027991236.1), cat (XP_003980687.1), cattle (NP_001193094.1), chimpanzee (XP_016809993.1), dog (XP_038536985.1), human (NP_291027.3), mouse (NP_663352.2), pig (XP_020939058.1), and rat (NP_001128209.1). (C) The *Trim41* expression profile between testicular cells based on published single-cell RNA sequencing data, visualized by 10 x genomics Loupe Browser. UMI means Unique Molecular Identifiers. (D) The *Trim41* expression profile between embryonic female germ cells based on published single-cell RNA sequencing data.

Fig. S2: Expression confirmation of *Clgn-Trim41 Tg*

(A) RT-PCR using postnatal testis of *Trim41* KO-Tg male mice. (B) Immunoblotting analysis with an anti-PA antibody. RT means reverse transcription. The black arrow indicates a specific band in Tg. (C) Immunoblotting analysis with an anti-TRIM41 antibody raised against a recombinant TRIM41 protein (35-85 amino acid residues). The black and red arrows indicate a specific band in Tg and WT, respectively.

Fig. S3: Immunostaining of zygotene–pachytene spermatocytes, related to Fig 3A-C.

(A and B) Additional immunostaining images of zygotene–pachytene spermatocytes

893 from Het (A) and KO (B) male mice. The white dashed line shows nuclei of different
894 cells. The white arrowhead indicates SYCP3 overloading. The images were arranged in
895 the order of meiotic progression from the zygotene stage (top) to the early pachytene
896 stage (bottom).

897

898 **Fig. S4: Immunostaining of meiotic cohesins.**

899 (A and B) SYCP3/REC8 immunostaining of spread nuclei from prophase spermatocytes
900 collected from adult WT (A) and KO (B) male mice. (C and D) SYCP3/RAD21L
901 immunostaining of spread nuclei from prophase spermatocytes collected from adult WT
902 (A) and KO (B) male mice.

903

904 **Fig. S5: Phenotypical analysis of *Trim41* KO females**

905 (A) Gross morphology of female gonads from E15.5 fetus. (B) Immunostaining of female
906 gonad sections. White arrows indicate intense SYCP3 signals. (C) Ovarian histology of
907 *Trim41* Het and KO females. The ovaries were collected 10 h after intraperitoneal
908 administration of hCG.

909

910 **Fig. S6: Immunostaining of prophase I spermatocytes, related to Fig 6A and B.**

911 (A and B) Immunostaining of spread nuclei from prophase spermatocytes collected from
912 adult ΔR -Het (A) and ΔR -Homo (B) male mice. At least three male mice were analyzed.
913 (C) The percentage of each meiotic prophase stage in immunostained spread nuclei
914 samples in A and B. The numerical data is available in Table S3.

915

916 **Fig. S7: Additional images for ΔR ING-TRIM41 accumulation, related to Fig 6C-F.**

917 (A) SYCP3/HA immunostaining of zygotene–diplotene spermatocytes from ΔR -Homo
918 male mice. White and blue arrowheads indicate HA signals on and outside of SYCP3
919 axes, respectively. At least three male mice were analyzed. (B–E) SYCP3/TRIM41
920 immunostaining of pachytene spermatocytes from Het (B), KO (C), ΔR -Het (D), and
921 ΔR -Homo (E) male mice. White arrowheads indicate HA signals on SYCP3 axes. At
922 least three male mice were analyzed.

923

924 **Fig. S8: Additional images for SYCP1 and BRCA1 immunostaining, related to Fig**
925 **6G and H.**

926 (A) SYCP1/HA immunostaining of surface chromosome spread from ΔR -Homo testis.
927 Magenta and yellow arrowheads indicate HA signals on the SYCP1 positive/negative
928 boundary and the non-homologous axis interface, respectively. At least three male mice
929 were analyzed. (B) BRCA1/HA immunostaining of surface chromosome spread from
930 ΔR -Homo testis. Red arrowheads indicate HA signals on the BRCA1 negative part of
931 the X chromosome axes. At least three male mice were analyzed.

932

933 **Fig. S9: Immunoprecipitation for substrate identification.**

934 (A and B) Immunoprecipitation using an anti-TRIM41 antibody and WT testis lysate.
935 TRIM41 immunoblotting (A) validated the immunoprecipitation capability of the
936 anti-TRIM41 antibody and the success of immunoprecipitation experiments. Red and
937 green arrowheads indicate TRIM41 and SYCP3, respectively. (C and D)
938 Immunoprecipitation using an anti-TRIM41 antibody and ΔR -Homo testis lysate. HA
939 immunoblotting (C) validated the success of immunoprecipitation experiments. Black
940 and green arrowheads indicate HA- ΔR ING-TRIM41 and SYCP3, respectively. (E and F)

941 Immunoprecipitation using an anti-HA antibody and Δ R-Homo testis lysate. HA
 942 immunoblotting (E) validated the success of immunoprecipitation experiments. Black
 943 and green arrowheads indicate HA- Δ RING-TRIM41 and SYCP3, respectively. (G and
 944 H) Mass analysis of co-IPed elutes using anti-TRIM41 (G) and anti-HA (H) antibodies.
 945 Proteins with 0 spectra in KO lysate were extracted and summarized in the table. The
 946 whole proteomics data are available in Table S4. (I) Proteins detected more than twice
 947 in G and H were summarized.

948

949 **Table. S1: Primers and gRNAs used in this study.**

950

951 **Table. S2: Antibodies used in this study.**

952

953 **Table. S3: Numerical data that underlies graphs.**

954

955 **Table. S4: The quantitative value of mass spectrometry analysis.**

956

Figure1

Figure2

Figure 3

Figure 4

A

B

C

D

Figure5

Figure 6