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1 **Original article**

2 **Title: Genetic factors derived from the MRL/MpJ mouse function to maintain the**
3 **integrity of spermatogenesis after heat exposure**

4

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15

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20

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25

26 **ABSTRACT**

27 MRL/MpJ mice possess highly heat-shock resistant spermatocytes (HRS) in comparison with
28 C57BL/6 mice. This resistance depends on the MRL/MpJ-type loci at the 81 cM region of
29 Chromosome (Chr) 1 and the 40 cM region of Chr 11. To evaluate the functions of these loci in
30 detail, we examined the histopathological changes resulting from experimental cryptorchidism
31 or transient scrotal heat stress (SHS) in the testes of C57BL/6-based congenic strains
32 (B6.MRLc1, B6.MRLc11, and B6.MRLc1c11) carrying the MRL/MpJ-derived loci responsible
33 for HRS. Among cryptorchid testes from congenic strains, those in B6.MRLc1c11 mice showed
34 the highest heat resistance, indicating that the genetic interactions between MRL/MpJ-derived
35 HRS loci on Chrs 1 and 11 may be important for maintaining spermatogenesis under continuous
36 testicular hyperthermia. In contrast, immediately after SHS induction, germ cell loss via
37 apoptosis was inhibited in B6.MRLc11 and B6.MRLc1c11 mice, similar to that in MRL/MpJ
38 mice. However, this HRS phenotype was not observed in C57BL/6 or B6.MRLc1 mice.
39 Furthermore, testicular calcification due to long-term damage by SHS induction was inhibited in
40 all congenic strains in comparison with that in C57BL/6 mice, indicating that each
41 MRL/MpJ-derived locus on Chrs 1 and 11 acted independently to facilitate the recovery of
42 heat-induced testicular damage by inhibiting calcification. B6.MRLc11 and B6.MRLc1c11 mice
43 showed greater recovery in spermatogenesis than B6.MRLc1 mice 60 days after SHS induction.

44 Therefore, the MRL/MpJ-derived HRS locus on Chr 11 might play an important role in
45 recovery from heat stress damage. Based on these results, we concluded that MRL/MpJ-derived
46 loci on Chrs 1 and 11 regulate testicular heat sensitivity through different mechanisms.

47 **Introduction**

48 Male germ cells, or sperm, are produced in a cyclic and complex process called
49 spermatogenesis, which occurs within the seminiferous tubules of the testes. Mammalian
50 spermatogenesis is divided into three phases: mitotic proliferation of stem spermatogonia,
51 meiotic differentiation of spermatocytes, and transformation of spermatids into spermatozoa.
52 Germ cell differentiation is regulated and supported by somatic Sertoli cells, which line the
53 seminiferous tubules. In most mammals, the testes are located in the scrotum outside the main
54 abdominal cavity. The temperature within the scrotum is regulated by a specialized blood
55 supply that acts as a heat exchange system. Thus, scrotal temperature is kept lower than the core
56 body temperature (Ivell 2007). Disruption of this system has an adverse effect on
57 spermatogenesis in several species, including humans (Rockett *et al.* 2001; Garolla *et al.* 2013).

58 Many studies have shown that exposure of the testes to a high-temperature
59 environment (i.e., surgical induction of cryptorchidism or mild testicular heat exposure)
60 damages the seminiferous epithelium, resulting in germ cell loss by apoptosis and alterations in
61 Sertoli cell morphology and function (Yin *et al.* 1997; Lue *et al.* 1999; Guo *et al.* 2007; Li *et al.*
62 2013). The cells most sensitive to testicular heat stress are spermatocytes (at the leptotene,
63 zygotene, and pachytene stages during meiosis) and round spermatids (Blacksaw *et al.* 1973;
64 Parvinen 1973). Moreover, testicular heat stress impairs protein, DNA, and RNA biosynthesis

65 (Steinberger 1991), damages spermatocyte DNA, and disrupts chromatin packing in sperm
66 nuclei (Garolla *et al.* 2013; Paul *et al.* 2008). However, the molecular mechanisms responsible
67 for degenerative changes that occur during heat-induced testicular dysfunction remain largely
68 unknown.

69 We have reported several unique characteristics of the MRL/MpJ mouse testis, such as
70 the appearance of testicular oocytes (Otsuka *et al.* 2008), a lower testis to body weight ratio than
71 that in other inbred strains (Otsuka *et al.* 2010), metaphase-specific apoptosis of meiotic
72 spermatocytes (Kon *et al.* 1999; Kon & Endoh 2000; Namiki *et al.* 2003; Kon 2005), and
73 heat-shock resistant spermatocytes (HRS) (Kon & Endoh 2001; Kazusa *et al.* 2004). In addition,
74 MRL/MpJ mice exhibit regenerative wound healing, such as the closure of ear punches and
75 cardiomyocyte regeneration (Clark *et al.* 1998; Leferovich *et al.* 2001). The presence of these
76 phenotypes depends on the MRL/MpJ genetic background, which is derived from C57BL/6,
77 C3H/He, AKR/J, and LG/J mice (Murphy 1981). Using quantitative trait loci analysis of
78 experimental cryptorchidism, we found that MRL/MpJ-type Chromosomes (Chrs) 1 (81 cM
79 region) and 11 (40 cM region) contained the loci responsible for HRS (Namiki *et al.* 2005).
80 However, we also demonstrated that the locus on MRL/MpJ-type Chr 1 responsible for HRS
81 was insufficient to protect spermatocytes from heat-induced damage (Chihara *et al.* 2014).

82 Therefore, to clarify the mechanisms regulating testicular heat sensitivity, it is essential to assess
83 the genetic interaction between MRL/MpJ-type HRS loci on Chrs 1 and 11.

84 In the present study, we investigated the roles of the MRL/MpJ-type loci in testicular
85 heat-resistance by comparing the pathological features of the testis after continuous or transient
86 heat stress induction in C57BL/6, MRL/MpJ mice, and C57BL/6-based congenic mouse strains
87 carrying the MRL/MpJ-type Chrs 1 and/or 11. Although both HRS-related MRL/MpJ-type loci
88 (Chrs 1 and 11) were necessary to maintain spermatogenesis under continuous hyperthermia,
89 the MRL/MpJ-type locus on Chr 11 alone was sufficient to mitigate damage induced by
90 transient hyperthermia. Based on these results, we propose that MRL/MpJ-derived loci on Chrs
91 1 and 11 regulate testicular heat-resistance through different mechanisms.

92

93 **Materials and Methods**

94 **Animals**

95 C57BL/6 and MRL/MpJ mice were purchased from an animal-breeding company (Japan SLC,
96 Hamamatsu, Japan). The C57BL/6-background congenic mouse strain
97 B6.MRL-(*D1Mit202–D1Mit403*) carrying the telomeric region of MRL/MpJ-type Chr 1
98 (67.97–81.63 cM) (B6.MRLc1) was generated in our laboratory (Otsuka *et al.* 2010). Similarly,
99 we generated the C57BL/6-background congenic mouse strain

100 B6.MRL-(*D11Mit21–D11Mit212*) carrying the MRL/MpJ-type Chr 11 (25.94–54.34 cM)
101 (B6.MRLc11). Furthermore, by mating B6.MRLc1 mice with B6.MRLc11, we generated the
102 double congenic mouse strain B6.MRL-(*D1Mit202–D1Mit403;D11Mit21–D11Mit212*) carrying
103 both of the MRL/MpJ-type Chr 1 (67.97–81.63 cM) and Chr 11 (25.94–54.34 cM)
104 (B6.MRLc1c11). Each mouse was maintained under specific pathogen-free conditions. For care
105 and handling of experimental animals, we adhered to the Guide for the Care and Use of
106 Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine
107 (approved by the Association for Assessment and Accreditation of Laboratory Animal Care
108 International).

109

110 **Experimental cryptorchidism**

111 Adult (8- to 14-wk-old) male mice were anesthetized with an intraperitoneal injection of sodium
112 pentobarbital (40 mg/kg body weight) dissolved in 0.01 M phosphate-buffered saline (PBS), and
113 an abdominal incision was made. To induce unilateral cryptorchidism, the right testis was
114 manipulated through the inguinal canal into the abdomen by cutting the gubernaculum and
115 sutured to the abdominal wall via the fat-pad. On day 21 after the operation, both testes were
116 removed and the weight ratio of the right cryptorchid testis to the left intact testis was calculated.
117 Each testis was fixed overnight in Bouin's solution and embedded in paraffin.

118

119 **Heat treatment**

120 To examine the effects of heat stress on mouse testes, 12-wk-old male mice were subjected to a
121 single application of scrotal heat stress (SHS) of 43°C for 20 min. Briefly, after anesthesia with
122 an intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight) dissolved in 0.01
123 M PBS, the hind legs, tail, and scrotum containing the testes were immersed in a
124 thermostatically controlled water bath. Untreated mice were used as controls. After 20 min, each
125 animal was dried and returned to its cage. Mice were sacrificed at 24–72 h and 10–60 days after
126 SHS induction to assess the short-term and long-term effects on the testes, respectively. The
127 testes were dissected and immediately weighed. They were immersion-fixed in ice-cold 4%
128 paraformaldehyde (PFA) in 0.1 M phosphate buffer overnight, and then embedded in paraffin.

129

130 **Immunohistochemistry**

131 The testes were then sectioned (2 μ m), deparaffinized, and re-hydrated. For
132 immunohistochemistry, sections were incubated for 15 min at 105°C in buffered citrate (pH 6.0)
133 for antigen retrieval of the following proteins: stimulated by retinoic acid gene 8 (STRA8),
134 DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination
135 (DMC1), SRY (sex determining region Y)-box 9 (SOX9), gremlin 2 (GREM2), and noggin

136 (NOG). Samples were treated with methanol containing 0.3% H₂O₂ to eliminate endogenous
137 peroxidase. After blocking with normal serum, sections were incubated with rabbit anti-STRA8
138 (1:2000; Abcam, Cambridge, UK), goat anti-DMC1 (1:500; Santa Cruz Biotechnology, Santa
139 Cruz, CA, USA), rabbit anti-single-stranded DNA (ssDNA, 1:200; IBL, Fujioka, Japan), rabbit
140 anti-SOX9 (1:2000; Merck Millipore, Billerica, MA, USA), rabbit anti-GREM2 (1:50;
141 Proteintech, Chicago, IL, USA), and rabbit anti-NOG (1:450; Abcam) at 4°C overnight. Next,
142 the sections were treated with biotin-conjugated goat anti-rabbit IgG antibodies (SABPO kit;
143 Nichirei, Tokyo, Japan) or with biotin-conjugated donkey anti-goat IgG antibodies (1:100;
144 Santa Cruz Biotechnology) for 30 min at room temperature followed by streptavidin-peroxidase
145 complex (SABPO kit) for 30 min at room temperature. The sections were incubated with
146 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.006% H₂O₂ until the
147 stain developed and then counterstained with hematoxylin. For histological analyses, digital
148 images of each section were acquired using a BZ-9000 microscope (Keyence, Osaka, Japan).
149 The immunopositive cells were counted in 200–400 tubules per testis using BZ-II Analyzer
150 software (Keyence).

151

152 **Histopathology**

153 Paraffin sections were prepared from testes fixed in either Bouin's solution or 4% PFA, as

154 described above, for periodic acid-Schiff-hematoxylin or von Kossa staining. For von Kossa
155 staining, 5- μ m-thick sections were incubated with 5% silver nitrate solution under direct
156 sunlight for 60 min. The slides were then washed in water, incubated with 5% sodium
157 thiosulfate pentahydrate for 3 min, rinsed, and counterstained for 5 min with nuclear fast red.
158 Seminiferous tubules containing von Kossa-positive mineral deposits were counted in 200–400
159 tubules per testis.

160

161 **Reverse transcription and quantitative real-time PCR**

162 Total RNA was extracted from whole mouse testes using TRIzol reagent (Life Technologies).
163 Total RNA was then treated with Turbo DNase (Life Technologies) for DNA digestion, and
164 complementary DNA (cDNA) was synthesized via reverse transcription using ReverTra Ace
165 (Toyobo, Osaka, Japan) and oligo-dT (Life Technologies). Quantitative real-time PCR (qPCR)
166 analysis was performed using cDNA, gene-specific primers (Table 1), Brilliant III Ultra Fast
167 SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, USA), and a real-time
168 thermal cycler (MX 3000P; Agilent Technologies). The mRNA levels of the target genes were
169 normalized to those of actin, beta (*Actb*).

170

171 **Statistical analyses**

172 Results were expressed as the mean \pm standard error (SE), and statistical analyses were
173 performed using PASW Statistics for Windows, Version 18.0 (IBM SPSS, Chicago, IL, USA).
174 Nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) was used to compare
175 each strain. One-way ANOVA followed by Dunnett's test was used to compare changes
176 between a treatment group and the corresponding untreated group or differences between
177 C57BL/6 mice and the other strains. *P*-values less than 0.05 were considered statistically
178 significant.

179

180 **Results**

181 **The effect of continuous hyperthermia on spermatogenesis in mice**

182 To evaluate strain-specific differences in spermatogenesis under experimental
183 cryptorchidism, the weight ratio of the cryptorchid testis to the intact testis and their histological
184 features were investigated at 21 days post-surgery (Fig. 1). Unlike tubules in the intact testis
185 (Fig. 1A), germ cell loss was obvious in the cryptorchid testis of C57BL/6 mice, and the
186 seminiferous epithelium consisted of only undifferentiated spermatogonia and Sertoli cells (Fig.
187 1B). In accordance with a previous report (Kon & Endoh 2001), almost all tubules in the
188 cryptorchid testis of the MRL/MpJ mice contained numerous spermatocytes (Fig. 1C), while
189 spermatocytes were found to a greater or lesser extent in cryptorchid testes of congenic mouse

190 strains (Fig. 1D–F). The testicular weight ratio was significantly higher in B6.MRLc1,
191 B6.MRLc1c11, and MRL/MpJ mice than in C57BL/6 mice. Although B6.MRLc11 mice
192 showed a relatively higher testicular weight ratio than C57BL/6 mice, the values were
193 significantly lower than in MRL/MpJ mice (Fig. 1G). For histological analysis, we estimated
194 the number of remaining spermatocytes by calculating the percentage of tubule cross-sections
195 containing spermatocytes (Fig. 1H). Each congenic strain maintained significantly higher
196 numbers of spermatocytes than C57BL/6 mice, and B6.MRLc1c11 mice showed highest count
197 among congenic strains. However, MRL/MpJ mice showed significantly higher heat-resistance
198 than each congenic strain.

199

200 **The effect of transient hyperthermia on spermatogenesis in mice**

201 Figure 2 shows the absolute testis weight at multiple time points after SHS induction.
202 Previously, we identified the locus associated with metaphase-specific apoptosis of meiotic
203 spermatocytes on the telomeric region of MRL/MpJ-type Chr 1 (Namiki *et al.* 2003). We also
204 reported a mutation in exonuclease 1 (*Exo1*, Chr 1, 81.90 cM), encoding a protein important for
205 DNA repair, as a potential cause of metaphase-specific apoptosis of meiotic spermatocytes in
206 MRL/MpJ mice (Namiki *et al.* 2003; Namiki *et al.* 2004). As we previously reported,
207 B6.MRLc1 mice carry the MRL/MpJ-derived locus on Chr 1 responsible for metaphase-specific

208 apoptosis (Otsuka *et al.* 2010). Because B6.MRLc1c11 mice also carry the same
209 MRL/MpJ-derived locus, both B6.MRLc1 and B6.MRLc1c11 mice had smaller testes compared
210 with the other strains analyzed. After SHS induction, testis weight gradually decreased over
211 time, reaching the minimum weight 10 days after treatment in all strains. Although testis weight
212 gradually recovered 20 days after SHS induction, it remained markedly low even at 60 days
213 post-induction in C57BL/6, MRL/MpJ, and B6.MRLc1 mice (44.83 ± 0.01 %, 46.58 ± 0.03 %, and
214 41.83 ± 0.03 % compared with strain matched control groups, respectively). In contrast,
215 B6.MRLc11 and B6.MRLc1c11 mice showed greater recovery of testis weight at 60 days after
216 SHS induction (62.11 ± 0.02 % and 79.56 ± 0.03 % compared with strain matched control
217 groups, respectively).

218 ssDNA-positive apoptotic cells, mainly spermatocytes and round spermatids, were
219 abundant in all strains at 24 h after SHS induction, but they were rarely detected in untreated
220 C57BL/6 controls (Fig. 3A–F). A histological analysis revealed that the number of
221 ssDNA-positive cells peaked at 24 h after SHS induction and then decreased in all strains (Fig.
222 3G). Characteristically, at 24 h after SHS induction, the number of ssDNA-positive cells was
223 significantly lower in MRL/MpJ, B6.MRLc11, and B6.MRLc1c11 mice than in C57BL/6 mice,
224 and B6.MRLc1 mice had relatively higher numbers of apoptotic cells at 24 h and 48 h compared
225 to the other congenic strains.

226 Changes in the number of primary spermatocytes at each developmental stage were
227 examined by immunohistochemical analysis of STRA8 and DMC1 (Fig. 4 and Supplemental
228 Fig. 1). Consistent with the induction of apoptotic germ cells as shown in Fig. 3, the number of
229 STRA8-positive cells (preleptotene and early leptotene spermatocytes) significantly decreased
230 beginning 24 h after SHS induction in C57BL/6 and B6.MRLc1 mice (Fig. 4A). On the other
231 hand, STRA8-positive cells began to decrease significantly in B6.MRLc1c11 mice at 48 h and
232 in MRL/MpJ and B6.MRLc11 mice at 72 h. C57BL/6, MRL/MpJ, and B6.MRLc1 mice
233 maintained lower numbers of STRA8-positive cells up to 60 days after SHS induction compared
234 to untreated controls. Consistent with the changes in testis weight, the number of
235 STRA8-positive cells in B6.MRLc11 and B6.MRLc1c11 had recovered to normal levels at 60
236 days after SHS induction (Fig. 4A).

237 The number of DMC1-positive cells (leptotene and zygotene spermatocytes)
238 transiently decreased in C57BL/6 mice at 24 h after SHS induction compared to untreated
239 controls; however, the other strains did not show a similar significant decrease compared to
240 their respective controls (Fig. 4B). Of note, DMC1-positive cells significantly increased in
241 B6.MRLc11 and B6.MRLc1c11 mice testes until 72 h after SHS induction compared to
242 untreated controls (Fig. 4B). However, in all strains, including B6.MRLc11 and B6.MRLc1c11,
243 a significant loss of DMC1-positive cells was observed at 10 days after SHS induction. Similar

244 to STRA8-positive cells, the number of DMC1-positive cells recovered to normal levels only in
245 B6.MRLc11 and B6.MRLc1c11 mice at 60 days after SHS induction.

246

247 **Mouse strain differences in the onset of testicular calcification induced by transient**
248 **hyperthermia**

249 In accordance with our previous studies, upon long-term follow-up analysis after SHS
250 induction, C57BL/6 and MRL/MpJ mice developed remarkable testicular calcification (Fig. 5).
251 von Kossa-positive testicular calcification initially appeared at 72 h to 10 days after SHS
252 induction, and staining became obvious at 20 days after SHS induction in C57BL/6 and
253 MRL/MpJ mice (Fig. 5A, B, and F). In contrast, in all congenic strains, calcified tubules were
254 barely detectable, and partial recovery of spermatogenesis was observed at 20 days after SHS
255 induction (Fig. 5C–F). Moreover, the onset of calcification was significantly inhibited in all
256 congenic strains compared with C57BL/6 mice throughout the observation period. Although
257 calcified tubules were frequently observed in MRL/MpJ mouse testis at 60 days after SHS
258 induction, the incidence rate was significantly lower in MRL/MpJ mice than in C57BL/6 mice.

259 To assess damage to the seminiferous epithelium induced by SHS, the number of
260 Sertoli cells was evaluated by immunohistochemical analysis of SOX9, a Sertoli cell marker
261 (Fig. 6). The number of SOX9-positive cells in C57BL/6 and MRL/MpJ mice significantly

262 decreased beginning 20 days after SHS induction, coincident with the abrupt onset of
263 calcification (Fig. 6G). Despite the inhibition of calcification, the number of SOX9-positive
264 cells in B6.MRLc1 mice significantly decreased at 60 days after SHS induction. Furthermore,
265 detachment of Sertoli cells from the basement membrane was frequently observed in the tubules
266 of C57BL/6, MRL/MpJ, and B6.MRLc1 mice at 60 days after SHS induction (Fig. 6B–D,
267 arrows). In contrast, although some tubules in B6.MRLc11 and B6.MRLc1c11 mouse testes
268 contained detached Sertoli cells at 60 days after SHS induction (Fig. 6E and F, arrows), the
269 number of SOX9-positive cells significantly increased at 10 days or 10–60 days after SHS
270 induction, respectively (Fig. 6G).

271

272 **The expression of calcification-related factors in mouse testes exposed to transient heat**
273 **stress**

274 Previously, we reported that the activities of bone morphogenic protein 2 (BMP2) and
275 GREM2, an endogenous BMP antagonist, correlated with the incidence of heat-induced
276 testicular calcification (Chihara *et al.* 2014). Because *Grem2* localizes at 81.08 cM on murine
277 Chr 1, we focused on *Nog*, which encodes another cytokine that inhibits the BMP2 activity and
278 localizes at 54.34 cM on murine Chr 11 (Pardali & Ten Dijke 2012). Compared to untreated
279 control testes, *Bmp2* mRNA expression tended to increase in testes of all strains at 10 days after

280 SHS induction (i.e., during early onset testicular calcification in C57BL/6 and MRL/MpJ mice)
281 (Fig. 7A). In contrast, *Grem2* mRNA levels were significantly higher in B6.MRLc1 and
282 B6.MRLc1c11 mice than in C57BL/6 mice at 10 days after SHS induction (Fig. 7B).
283 Furthermore, *Nog* mRNA levels were significantly higher in MRL/MpJ, B6.MRLc11, and
284 B6.MRLc1c11 mice than in C57BL/6 mice at 10 days after SHS induction (Fig. 7C). Notably,
285 mRNA expression levels of *Grem2* and *Nog* were also significantly higher in the untreated
286 testes of congenic strains carrying MRL/MpJ-derived loci on Chrs 1 and 11 compared to
287 C57BL/6 mice (Fig. 7B and C), indicating a genetic difference in their expression.

288 We next analyzed the expression and localization of GREM2 and NOG proteins by
289 immunohistochemistry at 10 days after SHS induction (Fig. 7D). Consistent with the qPCR
290 results, GREM2 protein expression was weak in the seminiferous tubules of C57BL/6,
291 MRL/MpJ, and B6.MRLc11 mice. In contrast, more intense GREM2 expression was observed
292 as granular cytoplasmic aggregates in Sertoli cells of B6.MRLc1 and B6.MRLc1c11 mice at 10
293 days after SHS induction (Fig. 7D, arrows). Similarly, although NOG expression was weak in
294 the seminiferous tubules of C57BL/6 and B6.MRLc1 mice, increased granular expression was
295 detected in Sertoli cells of MRL/MpJ, B6.MRLc11, and B6.MRLc1c11 mice (Fig. 7D,
296 arrowheads).

297

298 **Discussion**299 **Heat-shock resistant spermatogenesis under continuous hyperthermia in mice**

300 Accumulating evidence indicates that spermatogenesis, and the development of
301 spermatocytes and round spermatids in particular, is sensitive to hyperthermia (Blackshaw *et al.*
302 1973; Parvinen 1973). Interestingly, spermatocytes in MRL/MpJ mice were relatively resistant
303 to heat stress, as previously demonstrated in experimental cryptorchidism (Kon & Endoh 2001;
304 Kazusa *et al.* 2004). The heat stress-resistance of MRL/MpJ mouse testes is thought to depend
305 on MRL/MpJ-type loci on Chrs 1 (81 cM region) and 11 (40 cM region) (Namiki *et al.* 2005).

306 In the present study, we evaluated the relative resistance to heat stress of
307 spermatogenesis under continuous hyperthermia in B6.MRLc1, B6.MRLc11, and
308 B6.MRLc1c11 congenic mouse strains by direct comparison to their background strains,
309 C57BL/6 and MRL/MpJ mice. In cryptorchid testes, all MRL/MpJ-derived congenic strains
310 maintained relatively stable spermatogenesis compared with C57BL/6 mice. It is noteworthy to
311 mention that B6.MRLc1c11 mice, a congenic strain carrying both MRL/MpJ-type HRS loci on
312 Chrs 1 and 11, showed significantly higher resistance to heat stress than the other two congenic
313 strains. These results indicated that genetic interactions between MRL/MpJ-derived HRS loci on
314 Chrs 1 and 11 may be important to maintain spermatogenesis under conditions of continuous
315 testicular hyperthermia. However, B6.MRLc1c11 mice did not fully mimic the spermatogenic

316 capacity of MRL/MpJ mice when subjected to continuous hyperthermia, suggesting the
317 existence of other HRS-related loci that reside outside the 81 cM region of Chr 1 and the 40 cM
318 region of Chr 11.

319

320 **Sensitivity of spermatogenesis to transient hyperthermia in mice**

321 In the present study, we investigated the time-dependent effects of SHS induction on
322 spermatogenesis in C57BL/6 mice, MRL/MpJ mice, and the three additional congenic strains.
323 Consistent with previous reports (Paul *et al.* 2008; Paul *et al.* 2009; Chihara *et al.* 2014),
324 significant germ cell loss due to apoptosis was observed in the days following SHS induction.
325 However, among the congenic strains, B6.MRLc11 and B6.MRLc1c11 mice inhibited apoptosis,
326 similar to MRL/MpJ mice. Furthermore, B6.MRLc11 and B6.MRLc1c11 mice maintained a
327 steady number of STRA8- (preleptotene and early-leptotene spermatocytes) and DMC1-positive
328 cells (leptotene and zygotene spermatocytes) in the initial days after SHS induction. Consistent
329 with previous findings (Chihara *et al.* 2014), neither inhibition of apoptosis nor protection of
330 spermatogenesis against SHS induction was observed in B6.MRLc1 mice. Collectively, these
331 results suggest that the MRL/MpJ-derived HRS locus on Chr 11 might play an important role in
332 mitigating heat stress damage, thus maintaining spermatogenesis after transient hyperthermia.

333

334 The onset of testicular calcification after transient hyperthermia in mice

335 In the present study, although testicular calcification was not readily observed after
336 experimental cryptorchidism of all strains, there were strain differences in the incidence of
337 testicular calcification after SHS induction. These results might reflect differences in the
338 damage induced to seminiferous epithelium between continuous and transient hyperthermia. At
339 present, the etiology of testicular calcification is unclear. However, it has been suggested that
340 extensive germ-cell degeneration is involved (Nistal *et al.* 1979; Vegni-Talluri *et al.* 1980;
341 O'Shaughnessy *et al.* 2009; Kyrönlähti *et al.* 2011; Chihara *et al.* 2013; Chihara *et al.* 2014).
342 Analysis of multiple mouse strains has shown that there may be a genetic basis for susceptibility
343 to heat-induced testicular calcification. Both C57BL/6 and MRL/MpJ mice were suggested to
344 have precipitating factors that led to testicular calcification (Chihara *et al.* 2014). In accordance
345 with our previous result, B6.MRLc1 mice showed significantly decreased instances of testicular
346 calcification after SHS induction. Thus, one of the genes that predisposes for calcification could
347 localize on the 67.97–81.63 cM region of murine Chr 1. Previously, we identified
348 MRL/MpJ-derived *Grem2*, which localizes at 81.08 cM on Chr 1, as a candidate factor for
349 inhibition of calcification (Chihara *et al.* 2014). GREM2 is a cytokine that acts as an
350 extracellular BMP antagonist (Sudo *et al.* 2004), suggesting BMP activity regulates the onset of
351 calcification after transient scrotal hyperthermia. Indeed, many recent studies have suggested

352 that BMP proteins play key roles in modulating not only bone calcification but also pathological
353 calcification (e.g. vascular calcification) (Mikhaylova *et al.* 2007; Nakagawa *et al.* 2010; Pardali
354 *et al.* 2012). Interestingly, in this study, we demonstrated that B6.MRLc11 and B6.MRLc1c11
355 mice showed significantly reduced testicular calcification after SHS induction. Therefore, we
356 speculated that another BMP-relating gene may localize on the 25.94–54.34 cM region of
357 murine Chr 11. Predictably, *Nog*, which encodes a BMP signaling inhibitor, localizes at 54.34
358 cM on murine Chr 11 (Pardali *et al.* 2012). After SHS induction, up-regulation and localization
359 of GREM2 was observed in the Sertoli cells of B6.MRLc1 and B6.MRLc1c11 mice but not
360 those of B6.MRLc11 mice. Conversely, expression of NOG was upregulated in the Sertoli cells
361 of B6.MRLc11 and B6.MRLc1c11 mice but not in B6.MRLc1 mice. These results strongly
362 suggest that MRL/MpJ-derived *Grem2* and *Nog* act independently as factors inhibiting
363 calcification by antagonizing BMP activity. Further studies on the phenotype of the testis in
364 transgenic mice overexpressing *Grem2* and *Nog* would be needed to validate this hypothesis.

365 Moreover, a high incidence of calcification in MRL/MpJ mouse testis suggested that
366 MRL/MpJ mice may also have a potent precipitating factor for testicular calcification that
367 resides outside the 67.97–81.63 cM region of Chr 1 and the 25.94–54.34 cM region of Chr 11.
368 Indeed, the Sertoli cells of heat stress-exposed MRL/MpJ mouse testes showed degenerative
369 changes (Fig. 6), indicating that the ability of MRL/MpJ mouse Sertoli cells to maintain

370 spermatogenesis might be impaired long-term after SHS induction. Despite calcification
371 inhibition, B6.MRLc1 mice showed significant Sertoli cell degeneration at 60 days after SHS
372 induction. This result reinforced the concept that the MRL/MpJ-type HRS locus on Chr 11 is
373 superior to the MRL/MpJ-type locus on Chr 1 in mitigating damage induced by transient
374 hyperthermia. However, whether MRL/MpJ-derived HRS loci and calcification inhibitors are
375 the same factors is unclear in the present study and requires further exploration.

376 In summary, the present study demonstrated that the cooperative effects of the
377 MRL/MpJ-derived HRS loci on Chrs 1 and 11 are necessary to maintain spermatogenesis under
378 continuous hyperthermia. By contrast, the MRL/MpJ-derived HRS locus on Chr 11, rather than
379 the locus on Chr 1, might protect seminiferous epithelium from damage induced by transient
380 hyperthermia. Based on these results, we hypothesize that MRL/MpJ-derived loci on Chrs 1 and
381 11 regulate testicular heat-resistance through different mechanisms. Further studies, including a
382 more detailed analysis of the results found in the present study, may provide new insights into
383 the mechanisms regulating testicular heat-sensitivity.

384

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389

390 **Conflict of interest statement**

391 The authors declare no conflict of interest.

392

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484 **Figure legends**

485

486 **Fig. 1. Heat-shock resistance in experimental cryptorchid testis of mice.**

487 (A–F) Periodic acid-Schiff-hematoxylin stained cross sections of testes from mice at 21 days
488 after the cryptorchidism operation. No histological abnormalities were observed in intact
489 C57BL/6 mouse testis (A). In the cryptorchid testis of C57BL/6 mice, germ cell are largely
490 absent, while Sertoli cells and undifferentiated spermatogonia survived (B). Germ cells, with the
491 exception of spermatids, were observed in the cryptorchid testis of MRL/MpJ mice (C). In the
492 cryptorchid testes of B6.MRLc1 (D), B6.MRLc11 (E), and B6.MRLc1c11 (F) mice,
493 spermatocytes survived in some seminiferous tubules. Scale bars = 50 μ m. (G) Cryptorchid to
494 intact testis weight ratio at 21 days after the cryptorchidism operation ($n \geq 5$). Values shown are
495 the mean \pm SE. * $P < 0.05$ versus another strain, non-parametric one-way ANOVA
496 (Kruskal-Wallis). (H) Incidence of seminiferous tubules containing spermatocytes at 21 days
497 after cryptorchidism operation. Tubules containing at least one spermatocyte were counted in
498 200–500 tubules per testis ($n \geq 5$). Values shown are the mean \pm SE. * $P < 0.05$ versus another
499 strain, non-parametric one-way ANOVA (Kruskal-Wallis).

500

501 **Fig. 2. Changes in testicular weight after induction of scrotal heat stress.**

502 Control, untreated control; h, hours after scrotal heat stress (SHS) induction; d, days after SHS
503 induction. $n \geq 3$. Values shown are the mean \pm SE.

504

505 **Fig. 3. Apoptosis in mouse testes after scrotal heat stress induction.**

506 (A–F) Appearance of ssDNA-positive cells in the seminiferous epithelium after scrotal heat
507 stress (SHS) induction. ssDNA-positive cells were infrequently detected in testes from untreated
508 C57BL/6 mice (A). At 24 h after SHS induction, large portions of seminiferous tubules from
509 C57BL/6 (B) and B6.MRLc1 (D) mice contained numerous ssDNA-positive cells, whereas
510 testes of MRL/MpJ (C), B6.MRLc11 (E), and B6.MRLc1c11 (F) mice contained fewer
511 apoptotic cells. Scale bars = 50 μ m. (G) Changes in the number of apoptotic cells in C57BL/6,
512 MRL/MpJ, B6.MRLc1, B6.MRLc11, and B6.MRLc1c11 mice after SHS induction. The total
513 number of ssDNA-positive cells was counted in 200–400 tubules per control testis and heat
514 shock-exposed testis ($n \geq 3$). h, hours after SHS induction; d, days after SHS induction. Values
515 shown are the mean \pm SE. * $P < 0.05$ versus C57BL/6 mice at the same time point, as
516 determined by one-way ANOVA followed by Dunnett's test.

517

518 **Fig. 4. The changes in spermatocyte composition in mouse testes after scrotal heat stress**
519 **induction.**

520 The changes in the numbers of STRA8-positive cells (A) and DMC1-positive cells (B) in the
521 testes of each mouse strain after scrotal heat stress (SHS) induction. Positive cells were counted
522 in 200–400 tubules per testis ($n \geq 3$). h, hours after SHS induction; d, days after SHS induction.
523 Values shown are the mean \pm SE. * $P < 0.05$ versus strain-matched controls, as determined by
524 one-way ANOVA followed by Dunnett's test.

525

526 **Fig. 5. The onset of calcification in mouse testes after scrotal heat stress induction.**

527 (A–E) von Kossa-stained cross sections of testes from C57BL/6 (A), MRL/MpJ (B), B6.MRLc1
528 (C), B6.MRLc11 (D), and B6.MRLc1c11 (E) mice at 20 days after scrotal heat stress (SHS)
529 induction. Most tubules in C57BL/6 and MRL/MpJ mice contained von Kossa-positive granules
530 (black), whereas granules were barely detectable in the testis of B6.MRLc1, B6.MRLc11, and
531 B6.MRLc1c11 mice. Scale bars = 50 μ m. (F) Changes in the incidence of von Kossa-positive
532 seminiferous tubules in testes from each strain after SHS induction. Seminiferous tubules
533 containing von Kossa-positive mineral deposits were counted in 200–400 tubules per testis ($n \geq$
534 3). h, hours after SHS induction; d, days after SHS induction. Values shown are the mean \pm SE.
535 * $P < 0.05$ versus C57BL/6 mice at the same time point, one-way ANOVA followed by
536 Dunnett's test.

537

538 **Fig. 6. Damage to Sertoli cells in mouse testes after scrotal heat stress induction.**

539 (A–F) Appearance of SOX9-positive cells in the seminiferous epithelium after scrotal heat
540 stress (SHS) induction. SOX9-positive cells were aligned along the basement membrane of
541 seminiferous tubules in untreated C57BL/6 mouse testes (A). At 60 days after SHS induction,
542 SOX9-positive cell detachment from the basement membrane (arrows) was concomitant with a
543 significant decrease in these cells was observed in tubules from C57BL/6 (B), MRL/MpJ (C),
544 and B6.MRLc1 (D) mice. Although detached, SOX9-positive cells (arrows) were observed in
545 some tubules from B6.MRLc11 (E) and B6.MRLc1c11 (F) mice at 60 days after SHS induction;
546 however, most SOX9-positive cells were aligned along the basement membrane of tubules.
547 Scale bars = 50 μ m. (G) Changes in the number of SOX9-positive cells in the testes of each
548 mouse strain after SHS induction. Positive cells were counted in 200–400 tubules, including
549 calcified tubules, per testis ($n \geq 3$). h, hours after SHS induction; d, days after SHS induction..
550 Values shown are the mean \pm SE. * $P < 0.05$ versus strain-matched controls, as determined by
551 one-way ANOVA followed by Dunnett's test.

552

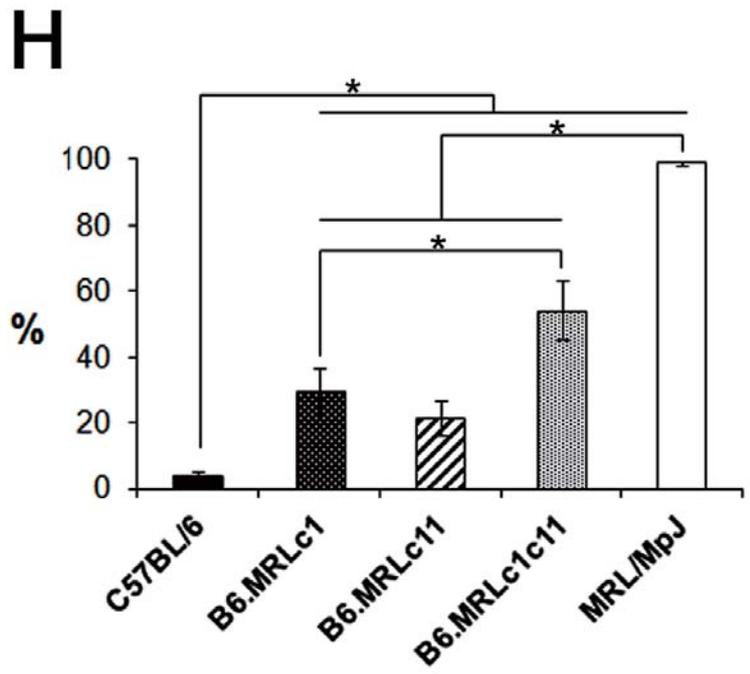
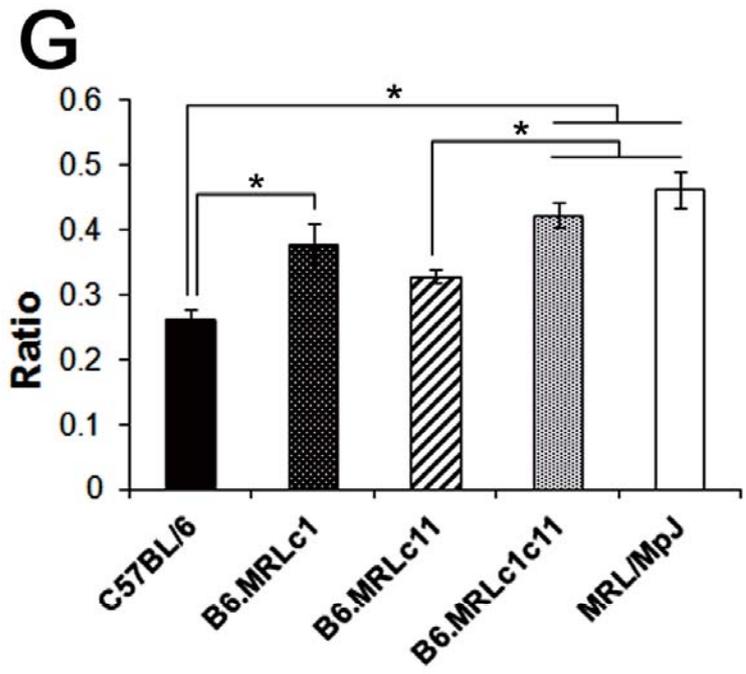
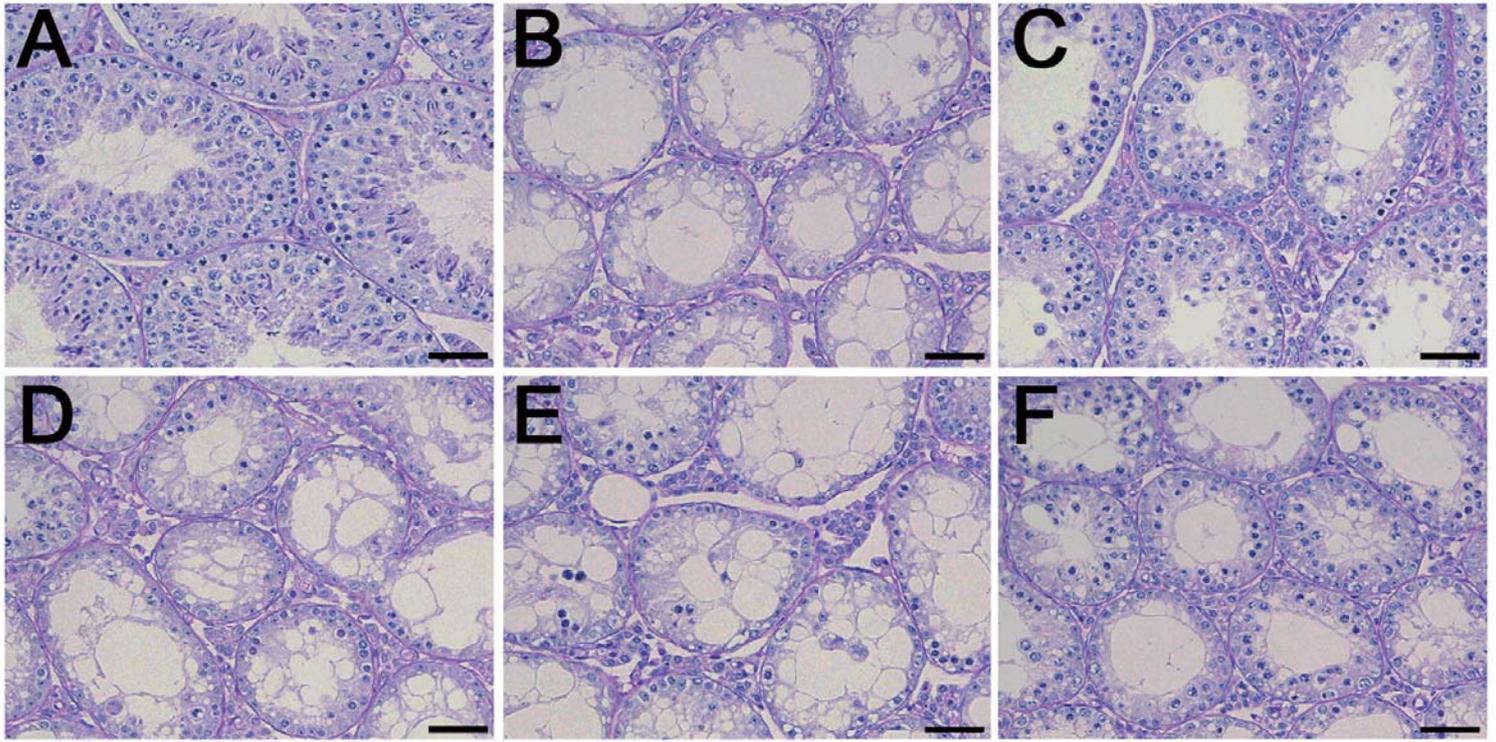
553 **Fig. 7. Expression of candidate calcification-regulatory molecules in mouse testes after**
554 **scrotal heat stress induction.**

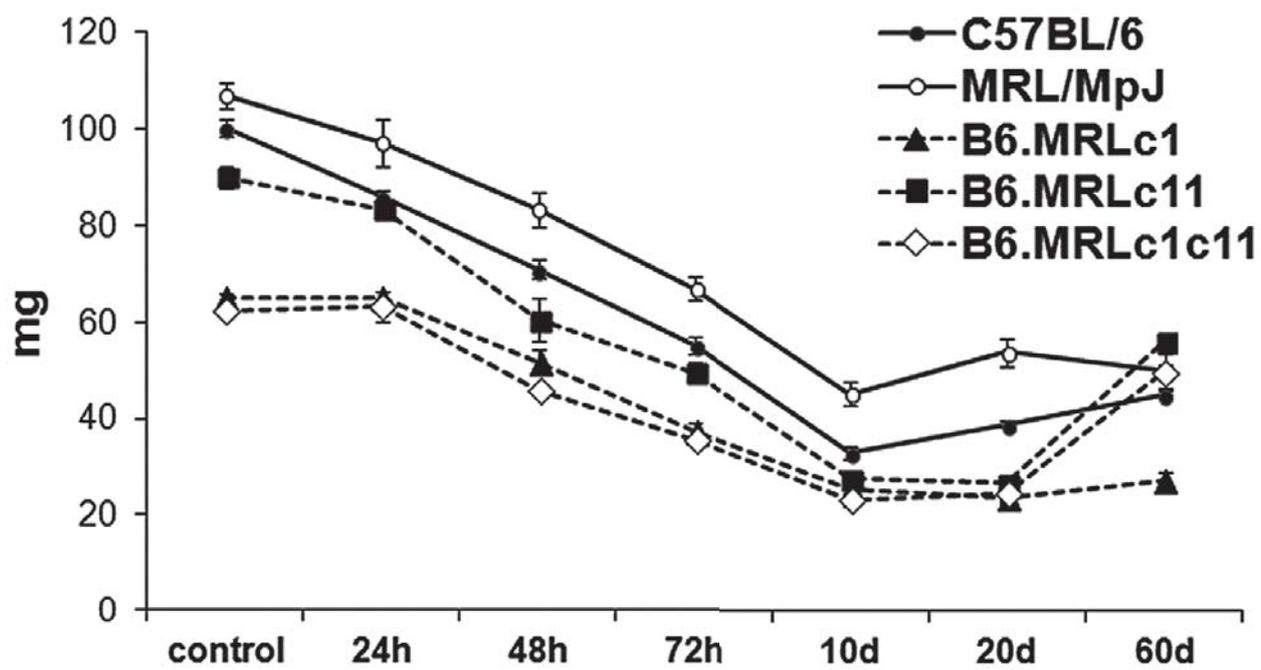
555 (A–C) qPCR results indicating changes in *Bmp2* (A), *Grem2* (B), and *Nog* (C) mRNA levels in
556 the testes of each mouse strain after scrotal heat stress (SHS) induction ($n \geq 3$). d, days after
557 SHS induction.. Values shown are the mean \pm SE. * $P < 0.05$ versus C57BL/6 mice at the same
558 time point; one-way ANOVA followed by Dunnett’s test. (D) Immunohistochemical analysis of
559 GREM2 and NOG proteins in the testes of C57BL/6, MRL/MpJ, B6.MRLc1, B6.MRLc11, and
560 B6.MRLc1c11 mice at 10 days after SHS induction. GREM2 proteins were identified as
561 granular cytoplasmic aggregates in the Sertoli cells of the B6.MRLc1 and B6.MRLc1c11
562 testes (arrows), whereas immunopositivity of GREM2 was weak in C57BL/6, MRL/MpJ, and
563 B6.MRLc11 mice. Similarly, NOG proteins were found as granular cytoplasmic aggregates in
564 the Sertoli cells of MRL/MpJ, B6.MRLc11, and B6.MRLc1c11 mice (arrowheads), whereas the
565 immunopositivity of NOG was weak in C57BL/6 and B6.MRLc1 mice. Scale bars = 10 μ m.

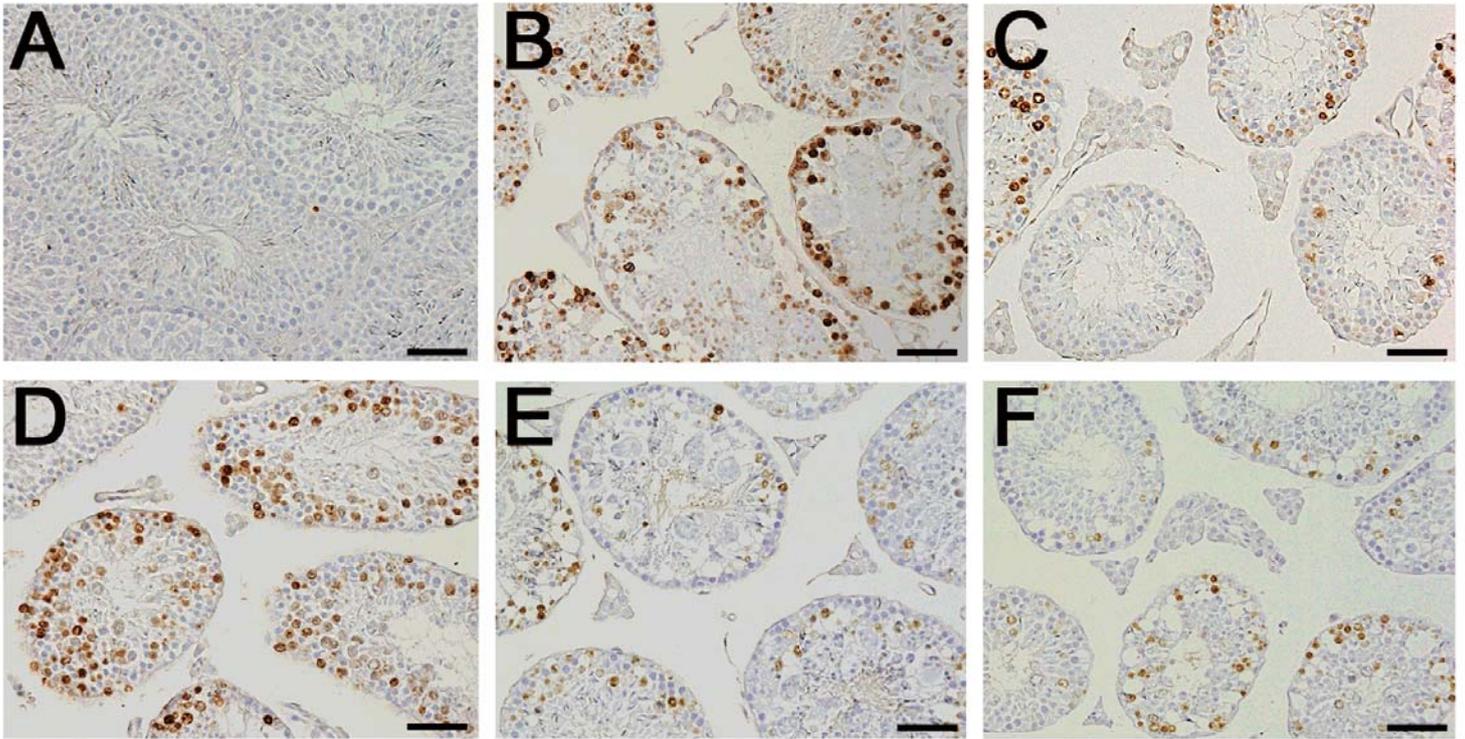
566 **Supplemental Figure Legend**

567 **Supplemental Figure 1. Appearance of spermatocytes at various meiotic stages in mouse**
568 **testes after scrotal heat stress induction.**

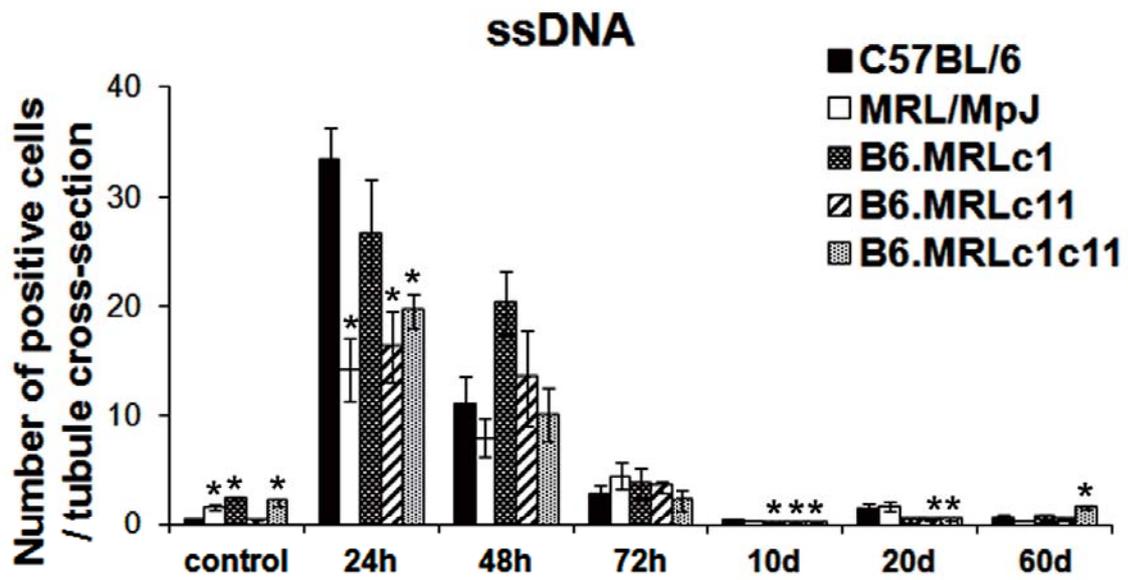
569 (A–F) The appearance of STRA8-positive cells in the seminiferous tubules. Cells positive for
570 STRA8 (preleptotene and early leptotene spermatocytes) were observed in a seminiferous
571 epithelial cycle-specific manner in untreated control testis from C57BL/6 mice (A). At 48 h
572 after scrotal heat stress (SHS) induction, STRA8-positive cells were barely detectable in
573 C57BL/6 (B) and B6.MRLc1 (D) mice, whereas they were abundantly present in MRL/MpJ (C),
574 B6.MRLc11 (E), and B6.MRLc1c11 mice (F). (G–L) The appearance of DMC1-positive cells
575 (leptotene and zygotene spermatocytes) in the seminiferous tubules. Tubules of testis from
576 untreated control C57BL/6 mice contained DMC1-positive cells in a cycle-dependent manner
577 (G). At 24 h after SHS induction, the number of DMC1-positive cells decreased, and DCM1
578 positivity weakened in C57BL/6 (H) and B6.MRLc1 (J) mice. In contrast, MRL/MpJ (I),
579 B6.MRLc11 (K), and B6.MRLc1c11 (L) mice maintained DMC1-positive cells. Scale bars = 50
580 μm .

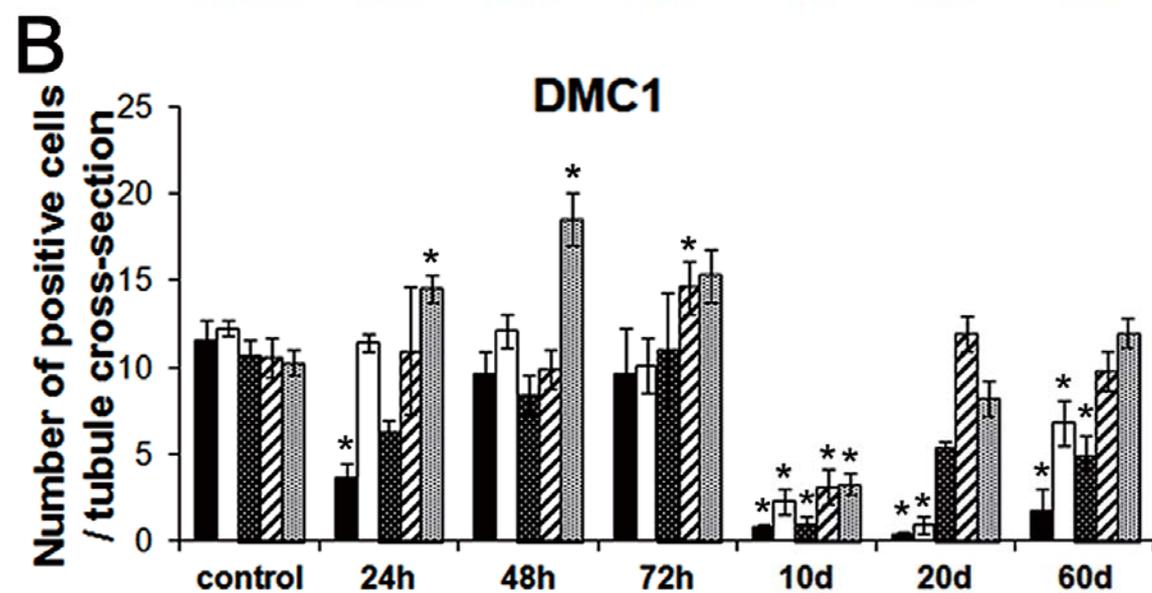
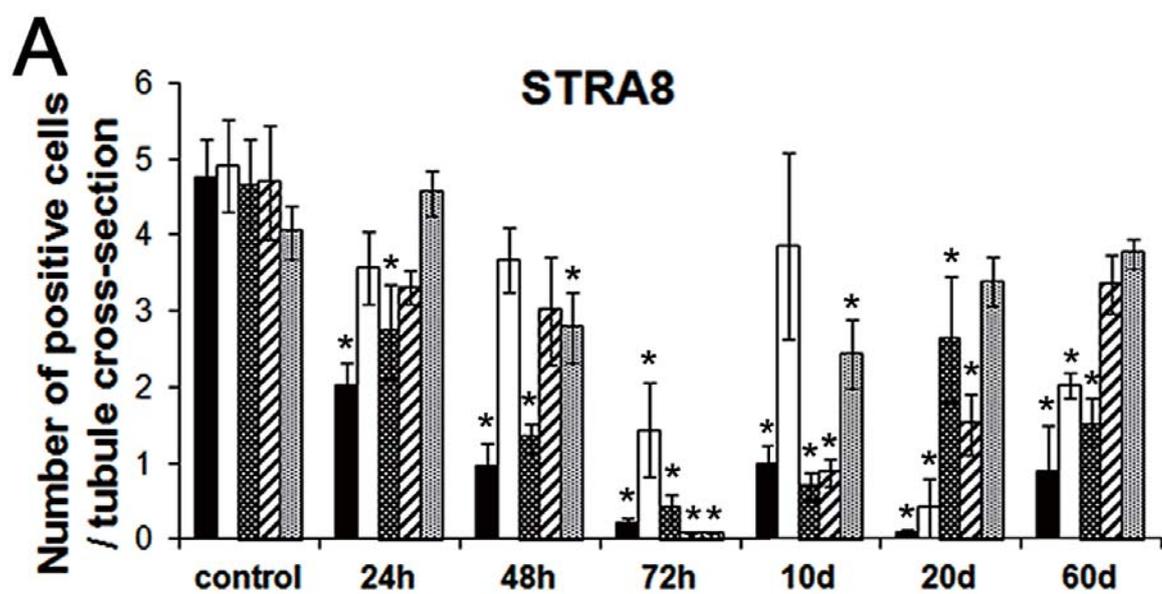




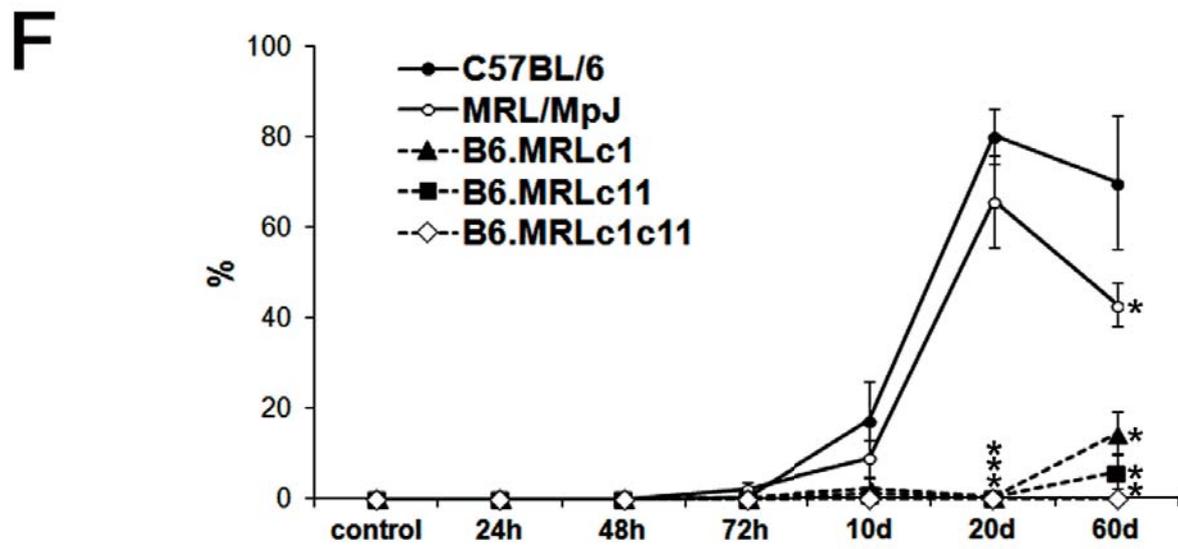
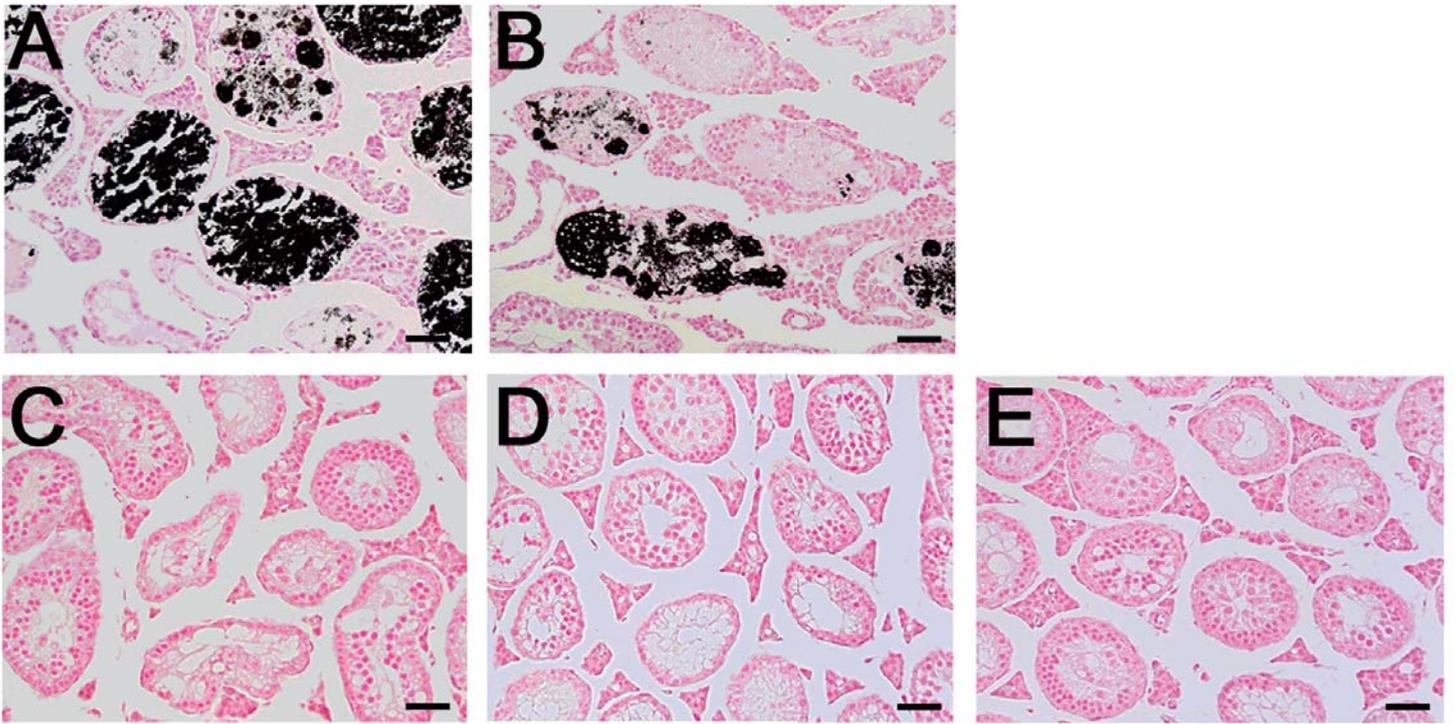


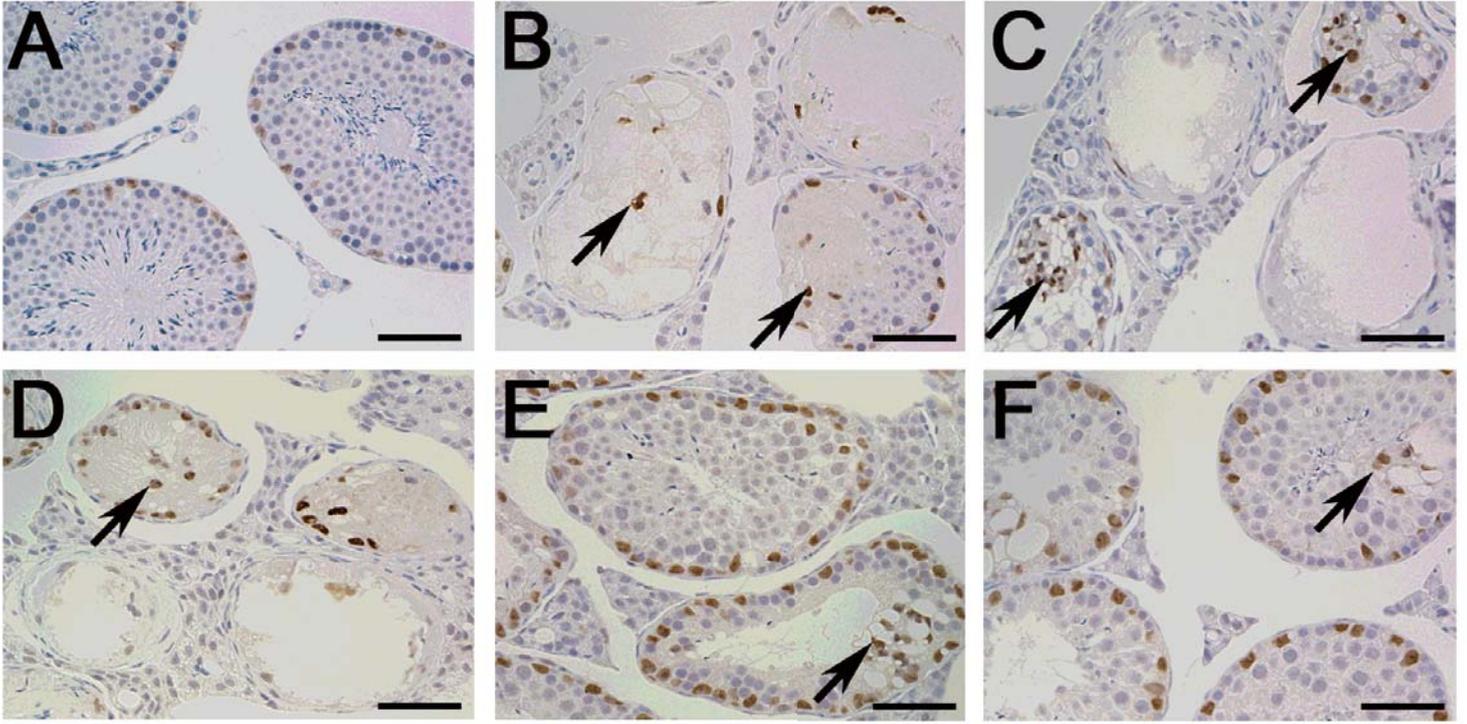
G





■ C57BL/6 □ MRL/MpJ ▣ B6.MRLc1 ▤ B6.MRLc11 ▥ B6.MRLc1c11





G

