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1 **Original paper**

2 **Immune-associated renal disease found in caspase 3-deficient mice**

3

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17

18 Running head: Renal pathology in *Casp3*-KO mice

19

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25 **Abstract**

26 Caspase (CASP) 3 is known as a representative effector CASP of apoptosis, and recently as a  
27 mediator in inflammatory cell death called pyroptosis. Interestingly, homozygotes of *Casp3*  
28 knockout (KO) mice with 129-background shows complete embryonic lethality, however, some  
29 of those with C57BL/6 (B6)-background (B6.129S1-*Casp3*<sup>tm1Flv</sup>/J) survived in lower rate (KO;  
30 11%, WT; 22%), developing immune abnormality-associated renal phenotypes. Homozygotes  
31 of *Casp3* KO mice with B6-background that survived for 8-12 months showed abnormality in  
32 the kidney and spleen but not in the other organs. Briefly, these *Casp3* KO kidneys showed  
33 proliferative glomerular lesions characterized by increased cells, matrices, immune-complex  
34 depositions containing IgA and complement 3 in the mesangial area, podocyte injuries, and  
35 inflammatory cell infiltrations in the tubulointerstitium. However, severe membranous lesion or  
36 renal dysfunction was not observed. Increased expression of inflammation-associated gene sets  
37 and inflammatory *Casps*, including *Casp12* was observed in these *Casp3* KO kidney. Moreover,  
38 these *Casp3* KO mice showed mild splenomegaly compared with WT mice. Thus, the long-  
39 surviving *Casp3* KO mice with B6-background developed renal lesions with altered immune  
40 conditions. CASP3 deficiency and aging factors could affect this phenotype by altering the  
41 function and/or development of each cell in the kidney and immune organs.

42

43 **Keywords**

44 Kidney, Glomerulus, Caspase 3, Knockout, Immunity

45

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49 (11–13 September 2018, Mr. Suzuki).

## 50 **Introduction**

51 In mammals, the caspase (CASP) family is a group of cysteine proteases constituting  
52 essential components of the apoptosis signalling pathway. Humans have CASP1-10, 12, and 14,  
53 whereas mice have CASP1-4, 6-9, 12, and 14 (Degterev et al. 2003; Ho and Hawkins 2005). In  
54 a study by Ho *et al* it was reported that at the initiation of cell death signalling at receptors such  
55 as TNF receptor superfamily member 6 (FAS) and TNF superfamily, member 6 (FASL) or death  
56 receptor activate the CASP2 and CASP8-9, which are called initiator CASPs. They transduce  
57 the apoptotic signal and subsequently activate the effector CASPs such as CASP3, 6, and 7,  
58 disassembling the cellular proteins (Ho and Hawkins 2005). A series of such biological process  
59 is known as CASP cascade, which strictly regulates cell death, especially apoptosis. The cells  
60 that are not needed during the developmental period and auto-reactive immune cells in the  
61 maturation processes of the immune system are mainly eliminated by apoptosis (Bouillet et al.  
62 2002; Fan et al. 2005). Further, defective cells damaged by ultraviolet rays or chemical  
63 substances are also removed by apoptosis (Roos and Kaina 2006). Thus, cell death pathway,  
64 particularly apoptosis, plays important roles in living bodies by removing specific cells to  
65 manage the adequate process of development and homeostasis.

66 CASPs are also involved in inflammatory cell death responses. Several mouse studies have  
67 revealed that pattern recognition receptors such as NLR family, pyrin domain containing 3  
68 (NLRP3) recognizes a specific stimulatory ligand, and NLRP3 binds to CASP1 and the adaptor  
69 protein, forms a protein complex, called inflammasome (Mariathasan et al. 2004; Fernandes-  
70 Alnemri et al. 2007). Then, CASP1 cleaves and activates the proinflammatory cytokines such  
71 as interleukin 1 beta (IL-1 $\beta$ ) and IL-18, which trigger an inflammatory reaction notifying  
72 surrounding cells of danger (Aachoui et al. 2013; Abderrazak et al. 2015; He et al. 2015).  
73 Moreover, in humans and mice, CASP1 can cleave gasdermin D (GSDMD), which promotes

74 inflammatory cell death, called pyroptosis (Gy and Broz). Pyroptosis is regulated by  
75 inflammatory CASPs such as CASP1, 4, and 5 in humans, and CASP1 and 4 in mice (Aglietti  
76 and Dueber 2017). In mice, CASPs activate GSDMD by cleaving its carboxy- and amino-  
77 terminal (N-terminal). The N-terminal of GSDMD forms a plasma membrane pore and increases  
78 permeability, causing inflammatory cell death (Kayagaki et al. 2015; Shi et al. 2015; Sborgi et  
79 al. 2016). Further, in mice, pyroptosis is mainly caused by intracellular invasion of pathogenic  
80 bacteria such as *Salmonella enterica* (Fink and Cookson 2007). Cell death induces pyroptotic  
81 cells to secrete inflammatory cytokines such as IL-1 $\beta$  and IL-6 and cause inflammation, which  
82 is useful for defence against pathogens (Aachoui et al. 2013; Shi et al. 2017).

83       Therefore, CASPs are closely associated with cell death as well as immunity. Further, their  
84 abnormalities are reported to be involved in some immune-associated refractory diseases.  
85 Briefly, CASP8-deficient mice lack Paneth cells and show reduced number of goblet cells in the  
86 ileum, and inflammatory lesions are formed at the same site, suggesting a central role of CASP8  
87 in gut mucosal immunity (Günther et al. 2011). These pathological features are similar to  
88 Crohn's disease in humans. In humans, it has been shown that the heterozygous mutation of  
89 CASP10 is responsible for autoimmune lymphoproliferative syndrome type II (Wang et al.  
90 1999). Thus, the pathological correlations between immune-associated diseases and genetic  
91 mutations were mainly found in initiator CASPs, but scarce in effector ones because their  
92 mutants died due to embryonic lethality in mice (Zheng et al. 1999). In mice and humans,  
93 CASP3 is one of the most popular effector CASPs, which is activated by initiator CASPs such  
94 as CASP8 (Fan et al. 2005). Then, it proceeds to the final stage of apoptosis by disassembling  
95 the intracellular target proteins. Therefore, CASP3 plays extremely important roles in  
96 development and cell death through apoptosis (Bergmann and Steller 2010; Porter and Ja 2015).  
97 Importantly, CASP3 causes pyroptosis by cleaving and activating gasdermin E which is related

98 to GSDMD in mice and humans (Gy et al. 2017; Wang et al. 2017). Thus, CASP3 plays  
99 important roles in apoptosis as well as in inflammatory responses, and its functional deficiency  
100 would be critical for cell death and the immune system. Interestingly, CASP3-deficient mice  
101 showing a unique phenotype associated with autoimmune abnormality was also discovered.

102 B6.129S1-*Casp3*<sup>tm1Flv</sup>/J (*Casp3* KO) mice were previously developed and used to analyse  
103 the role of CASP3 mainly in the developmental period (Kulda et al. 1996). Importantly,  
104 homozygotes of *Casp3* KO mice showed embryonic lethality with a certain probability, but upon  
105 careful investigation of the surviving homozygotes it was found that they showed unique kidney  
106 lesions and immune abnormalities. Since there is no basic and clinical evidence associated with  
107 CASP3 abnormality in the kidney and immune system organs, this study can justify the role of  
108 CASP3 as a disease-causing factor. This study raises a novel possibility for further elucidating  
109 the pathology of unknown refractory disease, especially focusing on the abnormalities between  
110 cell death and immunity.

111 **Materials and Methods**

112 *Animal ethics*

113 Animal experimentation was approved by the Institutional Animal Care and Use  
114 Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval No.  
115 15-0079, 16-0124). Experimental animals were handled in accordance with the Guide for the  
116 Care and Use of Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido  
117 University (approved by the Association for Assessment and Accreditation of Laboratory  
118 Animal Care International).

119

120 *Genotyping*

121 *Casp3* KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The  
122 genotyping polymerase chain reaction (PCR) amplification was performed according to the  
123 Jackson Laboratory protocol  
124 [https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5\\_MASTER\\_PROTOCOL\\_ID,P5\\_JRS](https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS)  
125 [\\_CODE:28532,006233](https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS), version June 14, 2017).

126

127 *Sample collection, serological analysis, and urinalysis*

128 Eight to twelve months old mice were used. Urine from each mouse was collected by  
129 applying pressure to the caudal area of the back and stored at -30 °C. Blood collected from  
130 femoral artery under the deep anaesthesia with the mixture of medetomidine (0.3 mg/kg),  
131 midazolam (4 mg/kg), and butorphanol (5 mg/kg) was centrifuged to obtain serum. All mice  
132 were euthanized by cervical dislocation. The spleen weight / body weight (SPW/BW) ratio was  
133 calculated. Serum concentrations of anti-double-stranded DNA (dsDNA) antibody and IgA were

134 measured by enzyme-linked immunosorbent assay (ELISA) using Lbis® Mouse Anti-dsDNA  
135 ELISA KIT, AKRDD-061 (Shibayagi Co. Ltd., Gunma, Japan) and ARG81183 Mouse IgA  
136 ELISA Kit (Arigo Biolaboratories Corp, Hsinchu, Taiwan) according to the manufacturer's  
137 instructions, respectively. To evaluate the renal function, the serum concentrations of blood urea  
138 nitrogen (BUN) and creatinine (CRE) were measured by Fuji Drichem (Fujifilm Medical Co.  
139 Ltd., Tokyo, Japan). Urinary concentrations of CRE and albumin (ALB) were measured using  
140 Urinary One-Step Creatinine Assay (Detroit R&D, Inc., Detroit, MI, USA) and A Murine  
141 Microalbuminuria ELISA (Exocell, Philadelphia, PA, USA) according to the manufacturer's  
142 instructions, respectively.

143

#### 144 ***Tissue preparation***

145 The systemic organs including spleen, kidney, salivary glands, thymus, liver, adrenal  
146 glands, testis, ovary, uterus, stomach, duodenum, jejunum, ileum, cecum, and colon were fixed  
147 with 4 % paraformaldehyde (PFA) at 4 °C overnight. For gene expression analysis, a part of  
148 kidneys was kept in RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA). For  
149 the renal histopathological analysis, kidneys from some of the animals were fresh-frozen in  
150 optimal cutting temperature (O.C.T.) compound (Diagnostic Biosystems, Pleasanton, CA, USA)  
151 using liquid nitrogen and isopentane for immunofluorescence. Kidneys from remaining animals  
152 were fixed by the combination of 2 % PFA and 2.5 % glutaraldehyde for 3-4 hours at 4 °C for  
153 transmission electron microscopy (TEM).

154

#### 155 ***Histopathological analysis***

156 Tissues fixed with PFA were processed and embedded in paraffin, cut in 3  $\mu\text{m}$  thick  
157 sections, and stained in haematoxylin-eosin (HE) staining. Moreover, the kidney sections were  
158 stained by periodic acid-Schiff (PAS), Masson's trichrome (MT), periodic acid-methenamine  
159 silver (PAM), and direct fast scarlet staining (DFS) staining.

160

### 161 *Immunostaining*

162 Fresh-frozen kidney sections were dried for 30 minutes and washed 3 times in phosphate-  
163 buffered saline (PBS). Paraffin sections (3  $\mu\text{m}$  thick) were deparaffinized followed by antigen  
164 retrieval. For immunohistochemistry, sections were soaked in methanol containing 0.3 %  $\text{H}_2\text{O}_2$   
165 for 20 minutes at room temperature for removal of endogenous peroxidase. After washing 3  
166 times with PBS, sections were incubated with the blocking serum for 1 hour at room temperature  
167 and primary antibodies overnight at 4  $^\circ\text{C}$ . After washing 3 times with PBS, sections were  
168 incubated with secondary antibodies for 30 minutes at room temperature and washed 3 times  
169 with PBS again. For immunofluorescence, sections were sealed with water-soluble encapsulant  
170 and observed under fluorescence microscopy (BZX-710, Keyence, Osaka, Japan). For  
171 immunohistochemistry, the sections were incubated with Streptavidin conjugated horseradish  
172 peroxidase [SABPO(R) kit; Nichirei, Tokyo, Japan] for 30 minutes and washed 3 times in PBS.  
173 For visualization of the positive reaction, the sections were incubated with 3,3' -  
174 diaminobenzidine tetrahydrochloride- $\text{H}_2\text{O}_2$  solution. The sections were slightly stained with  
175 haematoxylin. The details of antibody, antigen retrieval, and blocking are listed in  
176 Supplementary Table S1.

177

### 178 *TEM*

179 After fixation by the combination of 2 % PFA and 2.5 % glutaraldehyde for 3-4 hours at  
180 4 °C, samples were washed with 0.1 M phosphate buffer (PB) for 5 times, post-fixed with 1 %  
181 osmium tetroxide for 2 hours, and washed with 0.1 M PB 5 times. The samples were dehydrated  
182 with serially diluted ethanol, immersed in QY-1, and embedded into epoxy resin. Ultrathin  
183 sections (50 nm) were stained with uranium acetate and lead citrate, and observed under TEM  
184 (JEM-1210; JEOL, Tokyo, Japan).

185

### 186 *Histoplanimetry*

187 Fifty glomeruli were randomly selected from PAS stained kidney sections and their PAS-  
188 positive area for mesangial area, the total nuclear number, and glomerular size were measured  
189 in glomerulus by using BZ-X Analyzer (Keyence, Osaka, Japan). In 20 fields or in 20 glomeruli  
190 in tissue sections for immunohistochemical analysis, CD3- or B220-positive cells were  
191 enumerated. These parameters were compared between wild-type (WT) and KO mice.  
192 Abnormality was noted in these parameters of KO mice whereas WT mice showed normal  
193 parameters. The optimal cut-off value between normal and abnormal was analysed in each  
194 parameter by receiver operating characteristic (ROC) curve with JMP 13 (SAS Institute Japan  
195 Co., Ltd., Tokyo, Japan). Then, the ratio of normal and abnormal values was compared between  
196 WT and KO mice.

197

### 198 *RNA extraction and analysis*

199 Total RNA was extracted from kidney samples stored in RNAlater solution (Thermo Fisher  
200 Scientific) using the TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's  
201 instructions. The extracted total RNA was used as a template to synthesize complementary DNA

202 (cDNA) using ReverTra Ace® qPCR RT Master Mix (TOYOBO CO., LTD.; Osaka, Japan).  
203 Quantitative PCR (QPCR) analysis was performed on the cDNA using THUNDERBIRD®  
204 SYBR® qPCR Mix (TOYOBO CO., LTD.) and the respective gene-specific primers (See  
205 Supplementary Table S2). The QPCR analysis cycling conditions were: 95 °C for 1 min, 40  
206 cycles of 95 °C for 15 seconds, 60 °C for 45 seconds. Data were normalized by the values of  
207 beta-actin (*Actb*), and those of WT by using the delta-delta Ct method.

208

### 209 ***Microarray***

210 Four RNA samples were collected from each female WT and KO mice, and purified using  
211 the RNeasy® Micro Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol.  
212 Pooled one sample in each group was prepared, and the quality of RNA was determined by  
213 Agilent 2100 BioAnalyzer series II (Agilent, Santa Clara, CA, USA). The synthesis of cDNA  
214 and labelling of complementary RNA (cRNA) were performed by Low Input Quick Amp  
215 Labeling Kit (Agilent). Hybridization was performed by using Gene Expression Hybridization  
216 Kit and SurePrint G3 Mouse 8 x 60K ver.2.0 (Agilent). Scanning, quantification, and  
217 normalization were performed by Agilent Technologies Microarray Scanner (Agilent), Agilent  
218 Feature Extraction 12.0.3.1 (Agilent), and GeneSpring GX 14.9 (Agilent), respectively. For  
219 gene ontology (GO) analysis focusing on biological process, STRING (ELIXIR,  
220 Cambridgeshire, UK) was used.

221

### 222 ***Statistical analysis***

223 The results expressed as the mean ± standard error (SE) and analysed in the non-parametric  
224 manner. The significance between 2 groups was analysed by Mann-Whitney's *U*-test ( $P < 0.05$ ).

225 The correlation between 2 parameters was analysed using Spearman's rank correlation  
226 coefficient ( $P < 0.05$ ). For GO analysis, false discovery rate was applied.  
227

228 **Results**

229 ***Genotyping and protein expression of CASP3***

230 In PCR-based genotyping (Fig. 1a), WT and KO mice showed the bands of 320 and 300  
231 bp, respectively. Heterozygote (HT) mice showed both bands. During their breeding, the birth  
232 rate of homozygotes in *Casp3* KO mice, HT, and WT was 11% (11/101), 67% (68/101), and  
233 22% (22/101), respectively. Next, immunohistochemistry was performed to detect CASP3 in  
234 the collected organs (Fig. 1b and c). In WT mice, nuclear CASP3-positive reactions were  
235 observed in the thymus, spleen, and intestine. However, KO mice showed no reactions in these  
236 organs. In the kidney, no CASP3-positive cell was observed in both strains (Fig. 1d).

237

238 ***Glomerular lesions found in KO mice***

239 No histopathological change was observed in the systemic organs (nasopharyngeal parts,  
240 spleen, salivary glands, thymus, liver, adrenal glands, testis, ovary, uterus, stomach, duodenum,  
241 jejunum, ileum, cecum, and colon) checked (data not shown), except for the kidney of 8-12-  
242 month-old mice (Fig. 2a and b). Briefly, in the kidney of aged KO mice, the glomerular  
243 proliferative lesions including increased mesangial cells and matrixes were observed diffusely  
244 in both sexes, and the severity of them differed in glomeruli. No renal lesion was noted in WT  
245 mice. For histoplanimetry (Fig. 2c), mesangial area and nuclear number in the glomerulus was  
246 significantly increased in KO mice compared to those in WT mice in both sexes ( $P < 0.01$ );  
247 however, only in the male group, glomerular size was significantly increased in KO mice  
248 compared to WT mice ( $P < 0.05$ ).

249 Next, percentage of dividing normal and abnormal glomeruli in each histological parameter  
250 was analysed using ROC curve at 8-12 months of age (Fig. 2d). The ratio of abnormal glomeruli  
251 in mesangial area and nuclear number was significantly higher in KO mice compared with WT

252 mice in both sexes ( $P < 0.01$ ). Only in the male group, abnormally large glomeruli were seen in  
253 KO mice compared to those in WT mice ( $P < 0.05$ ).

254

### 255 *Characteristics of glomerular lesions in KO mice*

256 As shown in Fig. 2B, pathological assessments were carried out for female glomeruli at 8–  
257 12 months of age because female glomeruli tended to show the higher values than male  
258 glomeruli. As shown in Fig. 3a and b, for the MT staining in females, the aniline blue-stained  
259 area in the glomerulus, indicating the sclerotic area, was slightly increased in KO mice compared  
260 to WT mice. Further, PAM and DFS staining revealed that there is no glomerular membranous  
261 lesion and amyloid deposition in the glomerulus of both strains. Immunofluorescence staining  
262 using fresh frozen sections (Fig. 3c and d) revealed that global IgA- and complement 3 (C3)-  
263 positive reactions were more strongly observed in the mesangial regions of KO mice than WT  
264 mice, whereas there was no strain difference in those of IgG- and IgM-positive reactions.  
265 Ultrastructural observation under TEM revealed that high-electron dense materials were  
266 localized to the increased mesangial area in the glomerulus of KO mice, indicating the immune  
267 complex depositions (Fig. 3e).

268

### 269 *Glomerular cell injury and renal function in KO mice*

270 Next, the author investigated glomerular cell injuries at 8-12 months of ages. In  
271 immunofluorescence for podocyte markers including podocin, synaptopodin, and Wilms tumour  
272 1 homolog (WT1) in females, their positive area decreased in KO mice glomerulus compared to  
273 WT mice, particularly those of podocin and synaptopodin were localized to peripheral regions  
274 of glomerulus in KO mice (Fig. 4a and b). In TEM analysis, WT mice showed normal structure  
275 of podocytes, but podocyte foot process effacements were partially observed in KO mice (Fig.

276 4c). For renal functional analysis, the serum levels of BUN and CRE and urinary ALB/CRE  
277 ratio were not significantly different between WT and KO mice in both sexes (Fig. 4d).

278

### 279 *Altered immunity in KO mice*

280 From the IgA deposition in the glomerulus of KO mice (Fig. 3), the serum IgA level was  
281 analysed at 8-12 months of age, but statistical significance was not detected between both strains  
282 (Fig. 5a). Further, we also analysed the serum levels of anti-dsDNA antibody for autoimmune  
283 disease indices, and these tended to be higher in KO mice than in WT mice in both sex without  
284 significant differences. In the indices of systemic immune abnormalities, SPW/BW of KO mice  
285 was significantly higher than WT mice in both sexes ( $P < 0.01$ ).

286 In immunohistochemistry (Fig. 5b), CD3-positive T-cells were scarcely observed in the  
287 kidney of WT females, but they were abundantly detected in KO females, especially in their  
288 tubulointerstitium at 8-12 months of age. Compared to T-cells, B220-positive B-cells were  
289 relatively scarce in the both strains. In histoplanimetry, the number of CD3- or B220-positive  
290 cell in glomerulus did not differed between female WT and KO mice, but that in  
291 tubulointerstitium significantly increased in KO mice than in WT mice ( $P < 0.01$ ) (Fig. 5c).

292 Fig. 5d showed the gene expression levels of CASP family and apoptosis- or inflammation-  
293 associated molecules in the female kidney at 8-12 months of ages by QPCR analysis. For CASPs,  
294 the expression levels of inflammatory CASPs including *Casp1*, *Casp4*, and *Casp12* tended to  
295 be higher in KO mice than in WT mice, and the difference was significant for *Casp12* ( $P < 0.05$ ).  
296 Expression of apoptosis-related molecules including DNA fragmentation factor, alpha subunit  
297 (*Dffa*), TNF receptor superfamily member 6 (*Fas*), B cell leukemia/lymphoma 2 (*Bcl2*), BCL2-  
298 associated X protein (*Bax*), and Rho-associated coiled-coil containing protein kinase 1 (*Rock1*)

299 did not change between strains, but that of *Il1b* and tumor necrosis factor (*Tnf*) tended to be  
300 higher in KO mice than in WT mice. Significant difference was detected in *Tnf* ( $P < 0.05$ ).

301 We also comprehensively compared the gene expression in the kidney of WT mice and KO  
302 mice and performed the GO analysis. In the GO analysis using 2-fold upregulated genes in KO  
303 mice, 859 genes were detected, and Table 1 summarized the top 5 GO showing strong  
304 significance. In general, inflammation or immune-related biological process of GO including  
305 immune response (111 genes), immune system process (149 genes), defence response (103  
306 genes), innate immune response (65 genes), and immune effector process (57 genes), were  
307 detected. No GO was detected in the GO analysis using 2-fold downregulated genes in KO mice.

308

### 309 ***Qualitative histopathological analysis***

310 The correlation between the parameters of glomerular lesion (Fig. 2) and immune system  
311 abnormality (Fig. 5) was determined by Spearman's correlation coefficient as shown in Fig. 6.  
312 In all mice, the histopathological parameters for mesangial area and nuclear number of glomeruli  
313 but not glomerular size were significantly and positively correlated with the SPW/BW ratio and  
314 the serum levels of anti-dsDNA antibody ( $P < 0.01$ ). In the KO mice, the histopathological  
315 parameters for mesangial area (average, ratio) and nuclear number (average) were positively  
316 correlated with the serum levels of anti-dsDNA antibody ( $P < 0.01$ ). Also, the ratio of  
317 glomerular size was negatively correlated with the serum levels of anti-dsDNA antibody ( $P <$   
318 0.05).

319 **Discussion**

320 In the present study, novel phenotypes associated with kidney and spleen in the  
321 homozygotes of *Casp3* KO mice with C57BL/6 (B6)-background were found. Several previous  
322 studies reported that homozygotes of *Casp3* KO mice with B6-background showed a variety of  
323 phenotypes including neuronal apoptosis reduction and hyperplasia, hearing loss, and ovarian  
324 dysfunction (Kulda et al. 1996; Woo et al. 1998; Wang and Lenardo 2000; Matikainen et al.  
325 2001; Takahashi et al. 2001). Mainly, the developmental disorders of central nervous system  
326 (CNS) were critical for the survival of *Casp3* KO mice with B6-background, resulting in  
327 perinatal lethality (Kulda et al. 1996). Further, although *Casp3* KO mice with 129-background  
328 showed complete embryonic lethality, but those with B6-background could survive adulthood  
329 without obvious CNS defects with a low probability (Zheng et al. 1999), indicating that genetic  
330 factors, in addition to environmental, are essential to compensate or deteriorate CASP3  
331 deficiency. In qPCR analysis, the renal expression of effector *Casps*, such as *Casp6* and *Casp7*,  
332 did not significantly differ between WT and *Casp3* KO mice with B6-background, and these  
333 data suggested the presence of a compensatory pathway of CASP3 by the other CASP and/or  
334 CASP-independent signalling to survive through adulthood in *Casp3* KO mice with B6-  
335 background. Further, the effect of CASP3 deficiency varies depending on the mouse strain, it is  
336 necessary to analyse various *Casp3* KO mice lines carrying different genetic background to  
337 elucidate exacerbating or compensating gene factors for CASP3 deficiency and clarify the direct  
338 effect of it in kidney or immune system organs, which emphasizing the importance of CASP3  
339 deficiency in the pathogenesis of immune-associated kidney diseases.

340 These *Casp3* KO mice showed the renal inflammation, mild splenomegaly, and  
341 upregulation of the inflammatory gene sets, indicating their altered immune conditions due to  
342 CASP3 deficiency. Upregulation of inflammatory *Casps* such as *Casp1*, *4*, and *12*, and nuclear

343 factor  $\kappa\beta$  downstream molecules such as TNF in KO mice kidney indicates the exacerbated  
344 inflammation because of CASP3 deficiency. Since CASP3 is involved in the differentiation of  
345 immune progenitor cells in mice (Kuranaga and Miura 2007; Lamkanfi et al. 2007). CASP3  
346 deficiency is considered to affect the immune response or development of each resident cell,  
347 and the accumulation of these alterations, in addition to the effect of aging, would finally form  
348 the phenotype in each organ.

349 From glomerular pathological features of these *Casp3* KO mice, the author hypothesized  
350 the development of IgA nephropathy in KO mice. Representative human IgA nephropathy  
351 exhibits mesangial proliferation and IgA deposition to mesangial area accompanied by  
352 proteinuria and haematuria, associated with the upper respiratory inflammation that exists in the  
353 mucosa-associated lymphoid tissue (MALT) such as tonsil (Xie et al. 2004). However, in KO  
354 mice, renal function and serum IgA antibody level were similar to WT mice. Further, the  
355 morphology of MALTs in the intestine and nasopharynx did not differ between WT and KO  
356 (Supplementary Fig. S1). Podocyte injuries were suggested in KO mice, but the ALB/CRE did  
357 not significantly increase, indicating that lesions in KO mice were different from nephrotic  
358 syndrome based on glomerulosclerosis. Further, since KO mice showed mild splenomegaly and  
359 female-dominant glomerular damage, the author also considered the possibility of autoimmune  
360 glomerulonephritis like lupus nephritis. However, KO mice did not show remarkable glomerular  
361 membranous lesions and the significant increase of serum anti-dsDNA antibody level as found  
362 in the other model mice or human (Flores-Mendoza et al. 2018; Najafi et al. 2001). Thus, the  
363 glomerular pathological feature in KO mice did not completely overlap with representative  
364 known diseases, and they would develop specific pathological features due to the deficient of  
365 *Casp3* gene.

366 The correlation scores of mesangial proliferations and SPW/BW in all examined mice  
367 indicated a strong contribution of altered immunity to develop glomerular lesions in KO mice.  
368 Also, anti-dsDNA antibody level showed a strong correlation with the parameters of mesangial  
369 lesions, indicating its increasing as the result of disease progression. C57BL/6 strain usually  
370 developed age-related glomerular lesions from over 12 months of ages, including male-  
371 dominant sclerotic feature and immunocomplex depositions of IgG and IgM but not C3 (Linder  
372 et al. 1972; Yabuki et al. 2003, 2006). Therefore, aging is also considered as an important factor  
373 for causing glomerular lesions. Furthermore, KO mice at 6 months of age did not show clear  
374 glomerular lesions (Supplementary Fig. S2). On the other hand, renal lesions and immunological  
375 abnormalities of KO mice were more severe in females than males, indicating the effects of sex  
376 hormones (González et al, 2010). Although the direct effect of CASP3 deficiency to kidney  
377 resident cells was not clear in the present study, many *in vitro* studies using cultured mesangial  
378 cells reported the crucial role of CASP3 in the process of apoptosis (Mishra et al. 2005;  
379 Shimamura et al, 2003). Therefore, the difference of sensitivity to CASP3 deficiency in each  
380 kidney composing cell might also affect the development of glomerulus-specific lesions in mice.

381 The association between CASP deficiency and genetic disease has been reported previously  
382 in humans. Autoimmune lymphoproliferative syndrome (ALPS) is characterized by abnormal  
383 proliferation of lymphocytes due to cell death, and patients show symptoms of autoimmune  
384 disease, characterized by hepatosplenomegaly and lymph node swelling. The causes of ALPS  
385 include genetic abnormalities in FAS, the FAS ligand, and CASP10, which are involved in the  
386 FAS-dependent cell death (Martínez-Feito et al. 2016). Furthermore, a CASP8 deficiency results  
387 in similar symptoms (Rieux-Laucat et al. 2018). Although no study has assessed the CASP3  
388 deficiency in humans, our data potentially contribute to understanding the pathogenesis of  
389 CASP deficiency-related diseases to develop a novel therapeutic strategy.

390 In conclusion, the author found that survived and aged *Casp3* KO mice with B6-  
391 background exhibits the proliferative glomerular lesions characterized by the mesangial cell-  
392 associated lesions with mild sclerosis and the deposition of immune complexes including IgA  
393 and C3, and immune abnormality such as renal inflammation and altered spleen phenotypes.  
394 Since these phenotypes are specific compared with the other representative models or the  
395 patients in humans for immune-mediated renal diseases, the crucial pathological role of CASP3  
396 in the kidney composing cells was also suggested. The author concluded that CASP3 deficiency,  
397 in addition to the aging, would develop the characteristic glomerular lesions, and these findings  
398 emphasize the importance of CASP3 deficiency as a potential causative factor of immune-  
399 associated kidney diseases.

400

#### 401 **Compliance with Ethical Statements**

#### 402 **Conflict of Interest**

403 The authors have no conflicts of interest directly relevant to the content of this article.

404

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407

#### 408 **Ethical approval**

409 Animal experimentation was approved by the Institutional Animal Care and Use Committee of  
410 the Graduate School of Veterinary Medicine, Hokkaido University (approval No. 15-0079, 16-  
411 0124). Experimental animals were handled in accordance with the Guide for the Care and Use  
412 of Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University  
413 (approved by the Association for Assessment and Accreditation of Laboratory Animal Care  
414 International).

415

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491

492 **Author contributions**

493 T.S. and O.I. designed, performed experiments, and analysed data. T.N. analysed data. T.H.,  
494 Y.H.A.E. and Y.K. designed and reviewed the experiments. All authors were involved in writing  
495 the manuscript and provided final approval to publish the manuscript.

496

497

498 **Table 1. Gene ontology analysis in the kidney of *Casp3* KO mice.**

Pathway ID	Pathway description	Count in gene set	False discovery rate
<b>GO:0006955</b>	<b>Immune response</b>	<b>111</b>	<b>4.86E-54</b>
	<i>Aifl, Aim2, Apbb1ip, Batf, Bst2, Btk, C1qa, C1qb, C1qc, C1ra, C3, C4b, Card9, Ccl12, Ccl2, Ccl3, Ccl4, Ccl5, Ccl6, Ccl7, Ccl8, Ccl9, Ccr5, Ccr6, Ccr7, Cd180, Cd300a, Cd8a, Cfd, Cfp, Clec4d, Clec4e, Clec4n, Coro1a, Ctsc, Ctss, Cxcl10, Cxcl13, Cxcl9, Cybb, Ddx58, Dhx58, Dock2, Exo1, Fasl, Fcer1g, Fcgr1, Ffar2, Fgr, Fut7, Fyb, Gch1, Gzmb, H2-D1, H2-Q10, Hck, Ifih1, Ifit1, Ifit2, Ifit3, Iigp1, Il1rn, Il5ra, Irf1, Irf7, Irg1, Irgm1, Isg15, Kit, Lck, Lcp2, Lilrb3, Lst1, Ltf, Ly86, Mefv, Mmp7, Ms4a2, Mx2, Myo1f, Myo1g, Naip6, Oas1a, Oas2, Oas1l, Oas12, Pglyrp1, Pigr, Pik3cd, Pik3cg, Pou2f2, Rnf19b, S100a8, S100a9, Sh2d1a, Slc11a1, Star, Stat1, Stat2, Syk, Tlr1, Tlr7, Tlr8, Tnf, Tnfrsf1b, Trem2, Trim14, Tyrobp, Vav1, Vtn, Zbp1</i>		
<b>GO:0002376</b>	<b>Immune system process</b>	<b>149</b>	<b>6.43E-51</b>
	<i>AF251705, Adam8, Aifl, Aim2, Apbb1ip, Batf, Btk, Btla, C1qa, C1qb, C1qc, C1ra, C4b, C5ar1, Card11, Card9, Ccl12, Ccl2, Ccl3, Ccl4, Ccl5, Ccl6, Ccl7, Ccl8, Ccl9, Ccr5, Ccr6, Ccr7, Cd180, Cd300a, Cd300e, Cd300ld, Cd300lh, Cd3d, Cd8a, Cfd, Cfp, Chia, Clec4d, Clec4e, Clec4n, Coro1a, Ctsc, Ctss, Cxcl10, Cxcl13, Cxcl9, Cxcr3, Cybb, Ddx58, Dhx58, Dock2, Dock7, Emr1, Ercc2, Exo1, Fasl, Fcer1g, Fcgr1, Ffar2, Fgr, Fut7, Fyb, Gch1, Gfi1, Gzmb, H2-Q10, Hck, Hp, Ifih1, Ifit1, Ifit2, Ifit3, Iigp1, Ikzf1, Ikzf3, Il1rn, Il33, Il5ra, Il7r, Irf1, Irf7, Irg1, Irgm1, Isg15, Itga4, Itgax, Itgb2, Kit, Lck, Lcp2, Lilrb3, Lilrb4, Lst1, Ltf, Ly86, Mefv, Mfap5, Mmp7, Ms4a2, Mx2, Myo1f, Myo1g, Naip6, Nfam1, Oas1a, Oas2, Oas1l, Oas12, Pcd1, Pglyrp1, Pigr, Pik3ap1, Pik3cd, Pik3cg, Pilrb1, Pla2g2d, Plek, Pou2f2, Ptpn22, Ptprc, Rab33a, Rnf19b, Runx2, S100a8, S100a9, Selplg, Serpina3g, Sh2d1a, Slc11a1, Spib, Spn, Star, Stat1, Stat2, Syk, Tap1, Themis2, Tnf, Tnfrsf1b, Top2a, Trem2, Trem12, Trim14, Tyrobp, Vav1, Vcam1, Vtn, Zbp1</i>		
	<b>Defense response</b>	<b>103</b>	<b>7.20E-39</b>
	<i>Adam8, Agr2, Ahsg, Aifl, Aim2, Batf, Btk, C1qa, C1qb, C1qc, C1ra, C3, C4b, Card9, Ccl12, Ccl2, Ccl3, Ccl4, Ccl7, Ccl8, Ccr7, Cd180, Cd300a, Cd8a, Cfd, Cfp, Chi3l3, Chia, Clec4d, Clec4e, Clec4n, Coro1a, Cxcl10, Cxcl13, Cxcl9, Cxcr3, Cybb, Ddx58, Dhx58, Fcer1g, Fcgr1, Ffar2, Fgr, Gch1, H2-D1, Hck, Hp, Ifih1, Ifit1, Ifit2, Ifit3, Iigp1, Il33, Il5ra, Irf1, Irf7, Irg1, Irgm1, Isg15, Itgax, Kit, Lck, Ltf, Ly86, Lyz1, Lyz2, Mefv, Mmp7, Ms4a2, Mx2, Myo1f, Naip6, Nfkbid, Oas1a, Oas2, Oas1l, Oas12, Orm1, Orm2, Pglyrp1, Pik3cd, Pik3cg, Plac8, Ptprc, Rnf19b, Saa3, Sh2d1a, Slc11a1, Spn, Star, Stat1, Stat2, Syk, Tap1, Themis2, Tlr1, Tlr7, Tlr8, Tnf, Tnfrsf1b, Trem2, Trim14, Zbp1</i>		
	<b>Innate immune response</b>	<b>65</b>	<b>9.99E-33</b>
	<i>Aifl, Aim2, Bst2, Btk, C1qa, C1qb, C1qc, C1ra, C3, C4b, Card9, Ccl2, Ccl5, Cd180, Cfd, Cfp, Clec4d, Clec4e, Clec4n, Coro1a, Cybb, Ddx58, Dhx58, Fcer1g, Fcgr1, Fgr, Gch1, Hck, Ifih1, Ifit1, Ifit2, Ifit3, Iigp1, Irf1, Irf7, Irg1, Irgm1, Isg15, Lck, Ltf, Ly86, Mefv, Mx2, Naip6, Oas1a, Oas2, Oas1l, Oas12, Pglyrp1, Pik3cd, Pik3cg, Rnf19b, S100a8, S100a9, Slc11a1, Star, Stat1, Stat2, Syk, Tlr1, Tlr7, Tlr8, Trem2, Trim14, Zbp1</i>		
	<b>Immune effector process</b>	<b>57</b>	<b>3.33E-29</b>
	<i>Apbb1ip, Batf, Bst2, C1qa, C1qb, C1qc, C1ra, C3, C4b, Card9, Ccl3, Cd180, Cd300a, Cd8a, Cfd, Cfp, Clec4d, Clec4e, Coro1a, Ctsc, Cxcl10, Cxcl9, Ddx58, Dhx58, Dock2, Exo1, Fcer1g, Fcgr1, Fut7, Gzmb, Ifih1, Ifit1, Ifit2, Ifit3, Il33, Irf1, Irf7, Isg15, Itgax, Kit, Lilrb3, Mx2, Myo1f, Myo1g, Oas1a, Oas2, Oas1l, Oas12, Pou2f2, Ptprc, Rnf19b, Slc11a1, Syk, Tlr7, Tlr8, Tyrobp, Zbp1</i>		
Downregulated genes	NA		

---

62, 976 genes were examined. 859 genes were upregulated, and 441 genes were downregulated in the kidneys of KO mice compared with that in the kidneys of WT mice.

499

500

501 **Figure legends**

502 **Figure 1. Genotyping and caspase 3 protein expression in mouse organs.**

503 (a) Genotyping by polymerase chain reaction. Wild type (WT) mice showed a band of 320 bp  
504 whereas caspase 3 (*Casp3*) knockout (KO) mice showed a band of 300 bp. Heterozygotes  
505 (HT) mice showed bands of both 300 and 320 bp.

506 (b and c) CASP3 localization in the organs of WT and KO mice. Immunohistochemistry using  
507 females at 8 months. In WT mice, positive reactions were observed in thymus (panel b),  
508 spleen (panel b'), and epithelium or lamina propria of ileum (panel b''). There was no  
509 positive reaction in KO mice (panels c-c''). Arrowheads show positive cell. Bars = 50  $\mu$ m.

510 (d) CASP3 localization by immunohistochemistry in kidneys of 8-month-old mice. There was  
511 no positive reaction in renal glomerulus of WT and KO mice (panels d and d'). Bars = 50  
512  $\mu$ m.

513

514 **Figure 2. Glomerular pathology of caspase 3 knockout mice.**

515 (a and b) Glomerular features in wild type (WT) mice and caspase 3 knockout (KO) mice  
516 showing normal and abnormal morphology. Periodic acid-Schiff staining using mice at 8  
517 months. Compared with WT mice (panels a and b) and normal glomeruli of KO mice  
518 (panels a' and b'), abnormal glomeruli of KO mice showed increased nuclear number and  
519 mesangial area (panels a'' and b''). Bars = 50  $\mu$ m.

520 (c) Histoplanimetry for mesangial area (panel c), nuclear number (panel c'), and glomerular  
521 size (panel c'') using mice at 8-12 months. Values = mean  $\pm$  standard errors (SE). \*\* $P$  <  
522 0.01, \* $P$  < 0.05, Mann-Whitney  $U$ -test

523 (d) Ratio of normal and abnormal glomeruli for mesangial area (panel d), nuclear number  
524 (panel d'), and glomerular size (panel d'') using mice at 8-12 months. Threshold was

525 determined by receiver operating characteristic analysis of each glomerulus parameter.

526 Values = mean  $\pm$  SE. **\*\*** $P < 0.01$ , **\*** $P < 0.05$ , Mann-Whitney  $U$ -test.

527

528 **Figure 3. Characteristics of glomerular pathology in caspase 3 knockout mice.**

529 **(a and b)** Glomerular pathology of females at 8 months. Mild sclerosis was observed in caspase  
530 3 knockout (KO) mice (**panel b**), but not in wild type (WT) mice (**panel a**) in Masson's  
531 trichrome (MT) staining. No membranous lesion or amyloid deposit was observed in both  
532 WT (**panels a' and a''**) and KO mice (**panels b' and b''**) in periodic acid-methenamine silver  
533 (PAM) and direct fast scarlet staining (DFS). Square in PAM staining shows the normal  
534 glomerular basement membrane in KO mice. Bars = 50  $\mu$ m.

535 **(c and d)** Immunofluorescence for immune-complex detection using fresh frozen sections from  
536 females at 8 months. IgA- (**panel d**) and complement 3 (C3, **panel d''**)-positive reactions  
537 are observed in the mesangial area of KO mice, but not in WT mice (**panels c and c''**).  
538 Positive reactions for IgG and IgM are not observed in both mice (**panels c', c'''**, **d'**, and  
539 **d'''**). Bars = 50  $\mu$ m.

540 **(e)** Transmission electron microscopy (TEM) using females at 8 months. Increased mesangial  
541 cells and its area are observed in KO mice (**panel e**). Square area indicates that high-electron  
542 dense materials (showed by arrows) are observed in the mesangial area of KO mice (**panel**  
543 **e'**). Cap: capillary. Mes: mesangial cells. Bars = 2.0  $\mu$ m.

544

545 **Figure 4. Glomerular cell injury and renal function of caspase 3 knockout mice.**

546 **(a and b)** Peripherally altered reactions of podocin, synaptopodin, and Wilms tumor 1 homolog  
547 (WT-1) expression are observed in caspase 3 knockout (KO) mice (**panels b-b''**), but not

548 in wild type (WT) mice (panels a-a''). Immunofluorescence using females at 8 months.

549 Bars = 50  $\mu\text{m}$ .

550 (c) Transmission electron microscopy (TEM) using females at 8 months. Foot process  
551 effacements (showed by arrows) of podocytes are observed in KO mice (panel c'), but not  
552 in WT mice (panel c). Cap: capillary. Lu: lumens of Bowman's capsule. Bars = 2.0  $\mu\text{m}$ .

553 (d) Measurement of the serum blood urea nitrogen (BUN, panel d), serum creatinine (CRE,  
554 panel d'), the ratio of urinary albumin (ALB) to CRE (panel d'') using mice at 8-12 months.

555 Values = mean  $\pm$  standard errors (SE). Mann-Whitney *U*-test.

556

557 **Figure 5. Altered systemic and renal immunity in caspase 3 knockout mice.**

558 (a) Measurement of serum IgA antibody (panel a) and anti-double-stranded DNA (dsDNA)  
559 antibody levels (panel a') and the ratio of spleen weight (SPW)/body weight (BW, panel  
560 a'') in wild type (WT) and caspase 3 knockout (KO) mice using mice at 8-12 months.

561 Values = mean  $\pm$  standard errors (SE). \*\**P* < 0.01, Mann-Whitney *U*-test.

562 (b) Localizations of CD3- or B220-positive cells in the kidney. Immunohistochemistry using  
563 females at 8 months. Each positive cell is observed in caspase 3 knockout (KO) mice  
564 (panels b' and b'''), but few in wild type (WT) mice (panels b and b''). Bars = 100  $\mu\text{m}$ .

565 (c) Measurement of the number of CD3- or B220-positive cells in glomerulus (Glo, panels c  
566 and c') and tubulointerstitium (Ti, panels c'' and c''') using females at 8-12 months. Values

567 = mean  $\pm$  standard errors (SE). \*\**P* < 0.01, Mann-Whitney *U*-test.

568 (d) Gene expression analysis for caspase family (panel d), apoptosis, and inflammation (panel  
569 d') in the kidneys of females at 8-12 months. Quantitative PCR analysis. Values = mean  $\pm$

570 standard errors (SE). \**P* < 0.05, Mann-Whitney *U*-test.

571

572 **Figure 6. Correlation between glomerular histopathology and systemic immune**  
573 **alternation in caspase 3 knockout mice.**

574 **(a-c)** Correlations between the parameters of glomerular histopathology for mesangial area  
575 (panels a and a'), nuclear number (panels b and b'), and glomerular size (panels c and c')  
576 and spleen weight (SPW)/body weight (BW).

577 **(d-f)** Correlations between the parameters of glomerular histopathology for mesangial area  
578 (panels d and d'), nuclear number (panels e and e'), and glomerular size (panels f and f')  
579 and serum level of anti-double stranded DNA (dsDNA) antibody level.

580 \* $P < 0.05$ , \*\* $P < 0.01$ . Spearman's rank correlation coefficient. Black points: wild type  
581 (WT) mice. Red points: caspase 3 knockout (KO) mice. For statistical analysis, "All"  
582 included both sexes of WT (n = 8) and KO mice (n = 12), and "KO" included KO mice  
583 only. Black and red dotted lines indicated the approximate curves in "All" and "KO",  
584 respectively.

Figure 1

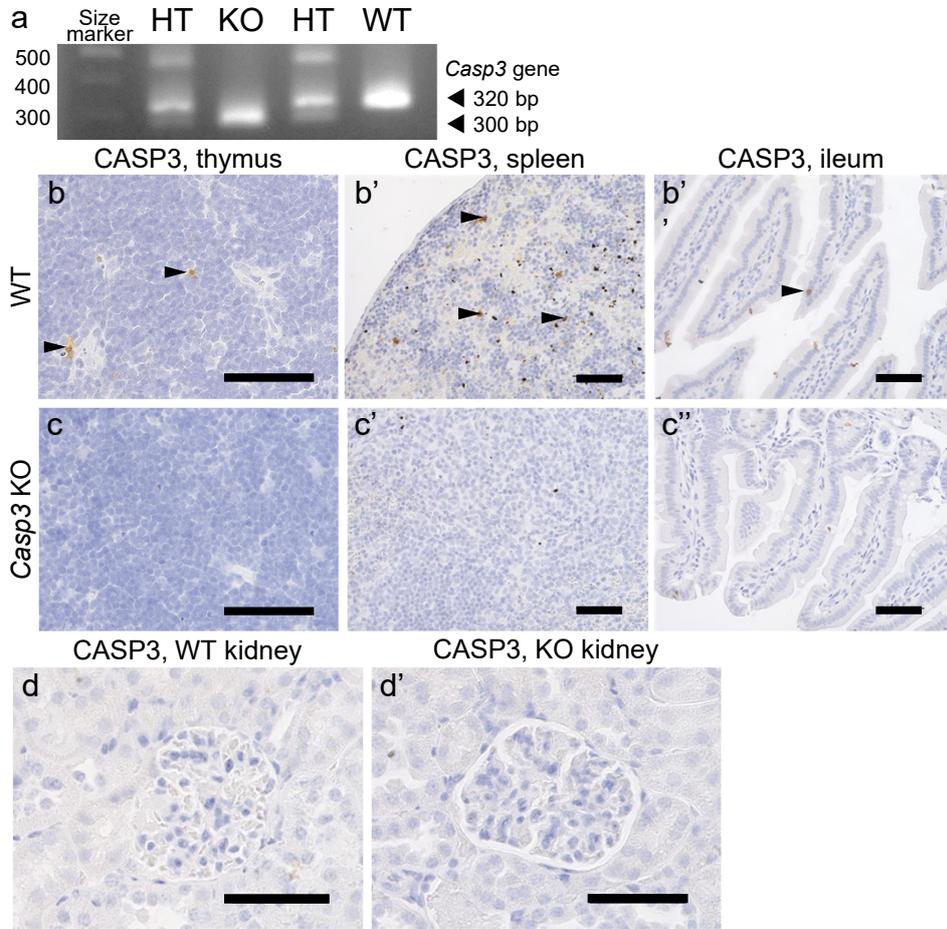


Figure 2

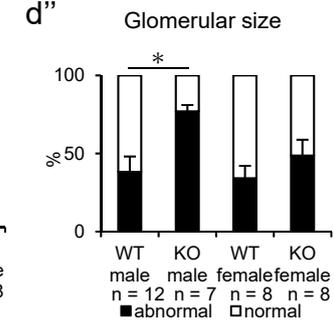
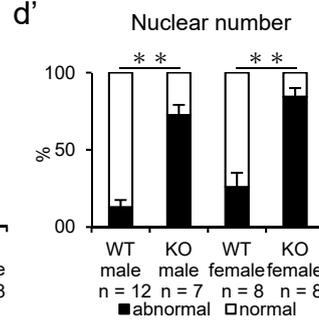
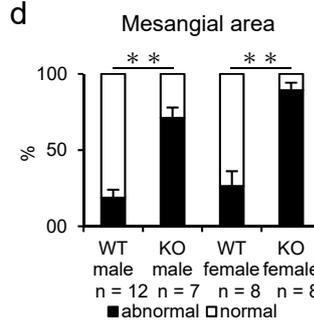
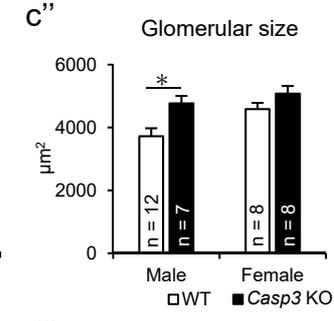
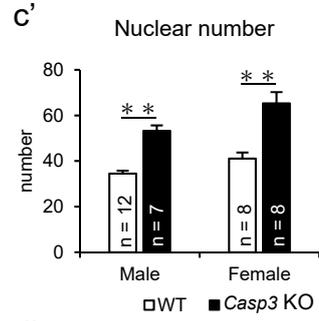
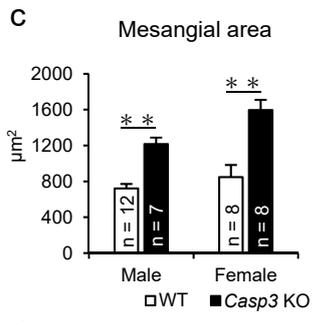
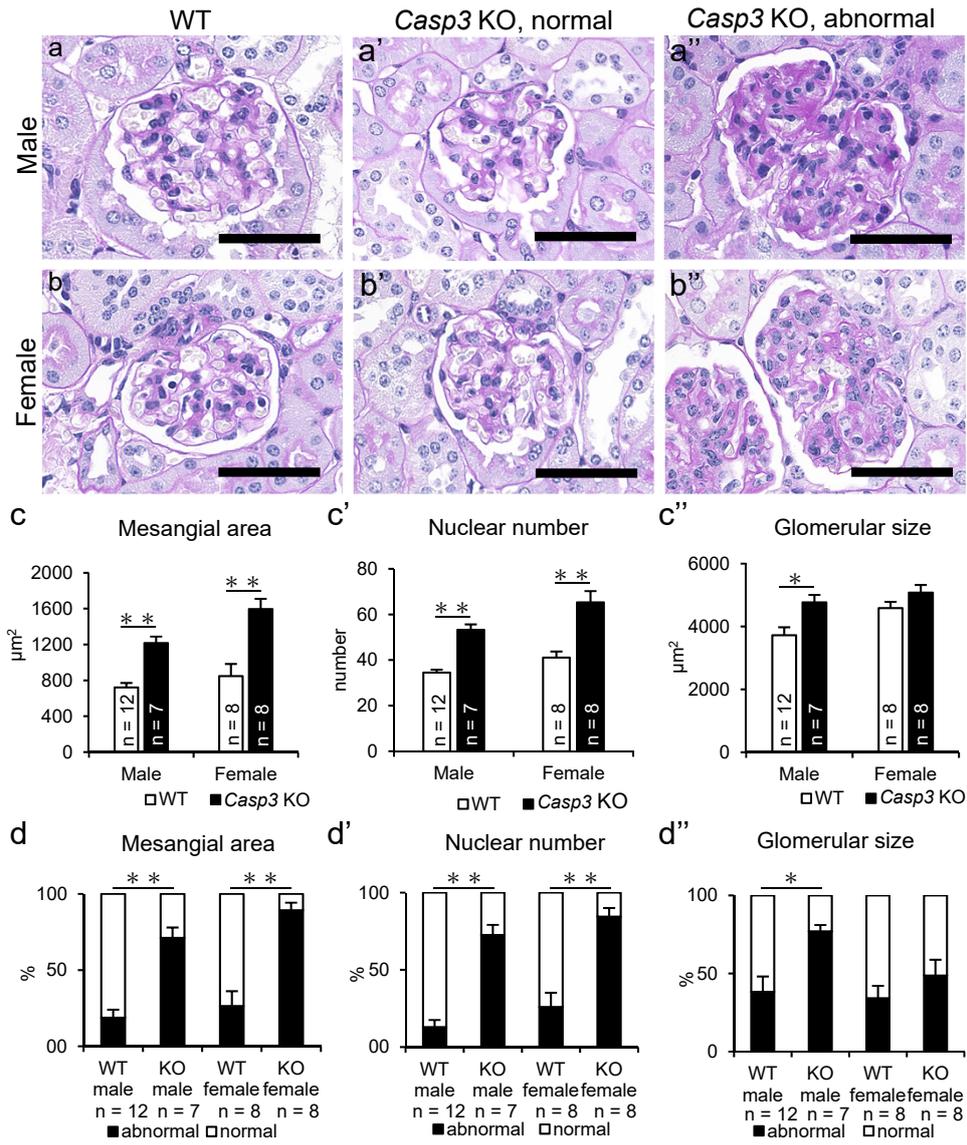


Figure 3

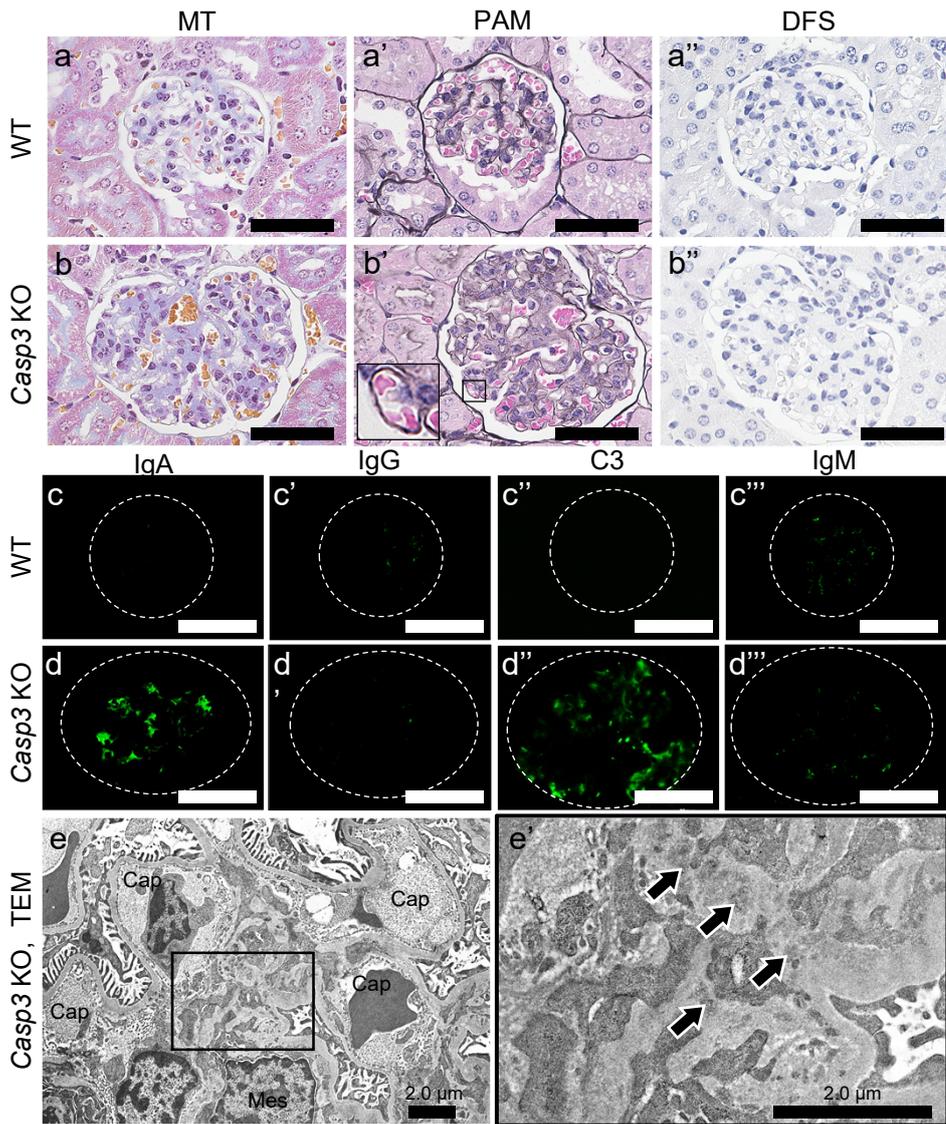
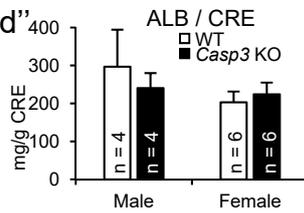
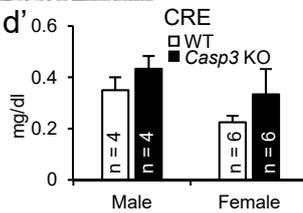
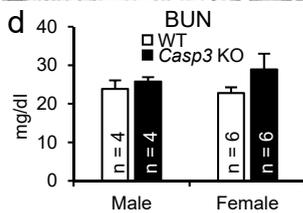
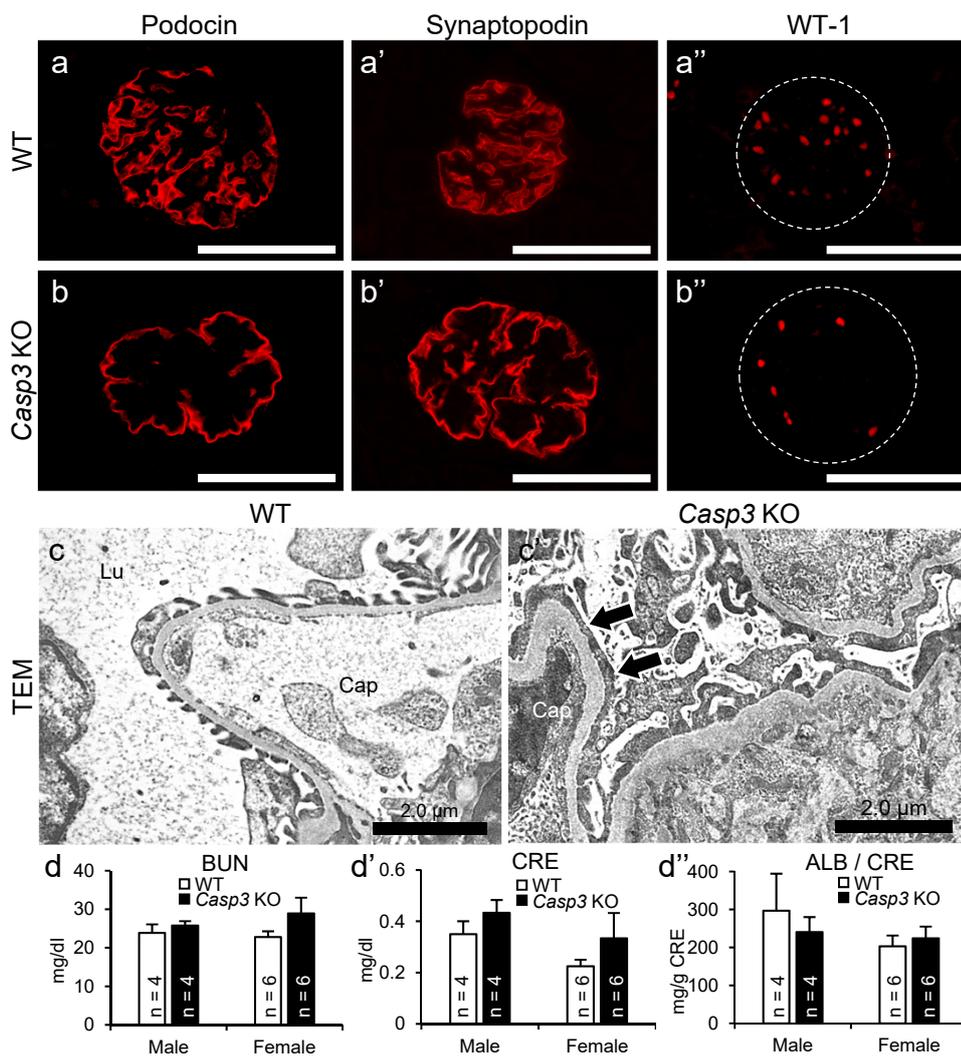


Figure 4



# Figure 5

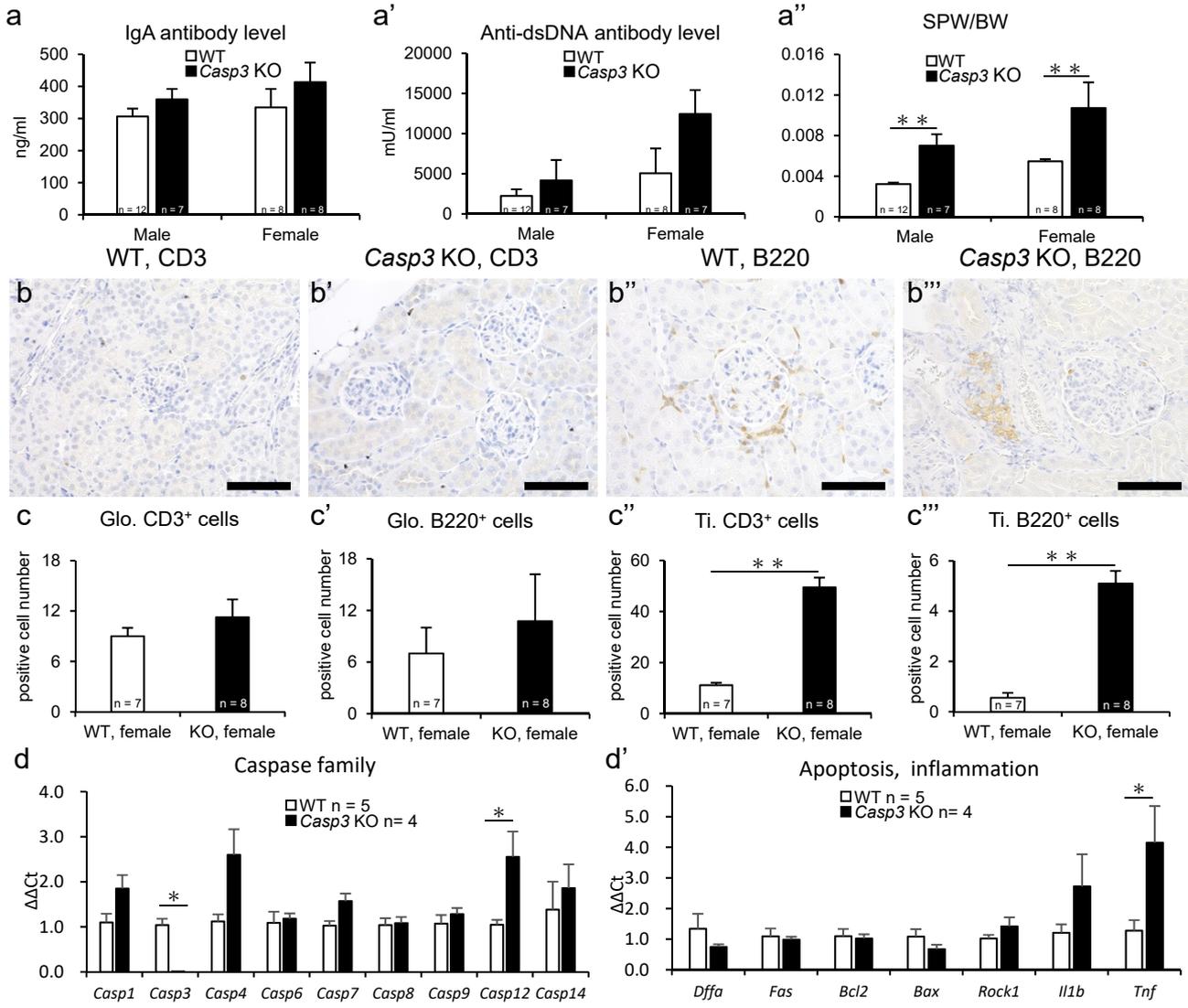
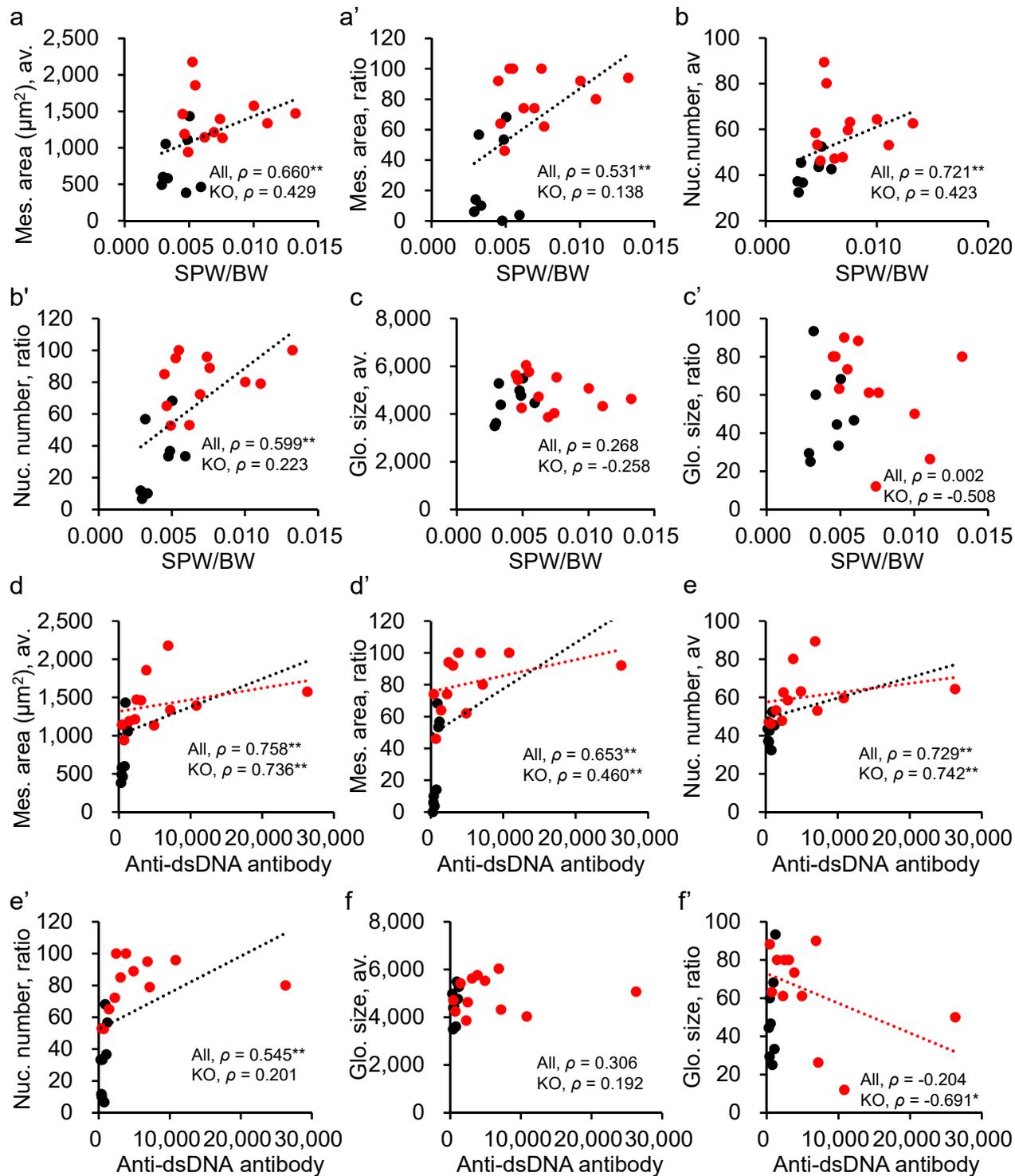
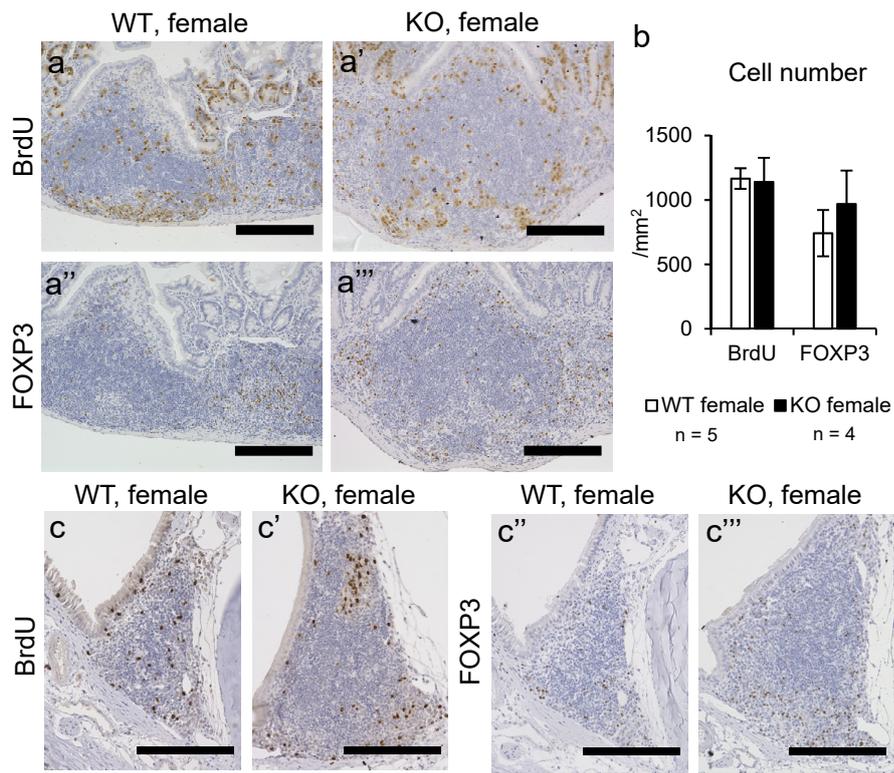


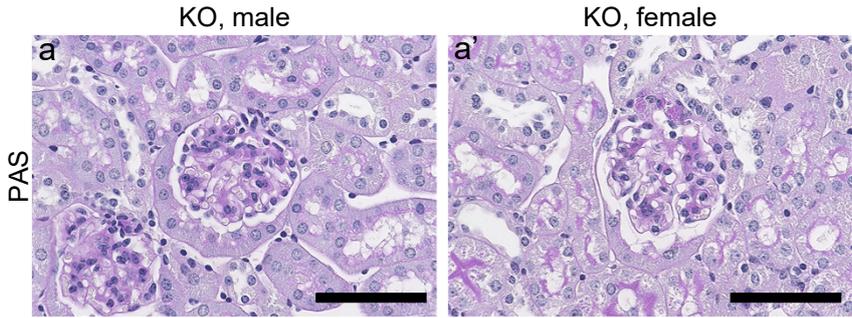
Figure 6



# Supplemental figure 1



# Supplemental figure 1



1 **Supplementary Table S1. Antibodies used in this study.**

<b>Antibody</b>	<b>Host</b>	<b>Dilution</b>	<b>Source</b>	<b>Retrieval</b>	<b>Blocking</b>	<b>Note</b>
CASP3	Rabbit	1:300	Cell Signaling Technology, Danvers, MA, USA	CB	10 % Goat normal serum	
CD3	Rabbit	1:200	Nichirei, Tokyo, Japan	Tris	10 % Goat normal serum	
B220	Rat	1:1600	Cedarlane, Burlington, Canada	CB	10 % Goat normal serum	
BrdU	Rat	1:400	Abcam, Cambridge, UK	CB	10 % Goat normal serum	Supplemental figure S1
FOXP3	Rat	1:400	Thermo Fisher Scientific, Waltham, MA, USA	Tris	10 % Goat normal serum	Supplemental figure S1
IgA	Rabbit	1:400	BETHYL laboratories, Montgomery, AL, USA	-	5 % Donkey normal serum	
IgG	Rabbit	1:400	BETHYL laboratories, Montgomery, AL, USA	-	5 % Donkey normal serum	
IgM	Rabbit	1:400	BETHYL laboratories, Montgomery, AL, USA	-	5 % Donkey normal serum	
C3	Rabbit	1:316	Abcam, Cambridge, UK	-	5 % Donkey normal serum	
Podocin	Rabbit	1:800	IBL, Gunma, Japan	CB	5 % Donkey normal serum	
Synaptopodin	Mouse	1:500	Fitzgerald Industries International, Acton, MA, USA	Tris	5 % Donkey normal serum	
WT-1	Rabbit	1:200	Santa Cruz Biotechnology, Dallas, TX, USA	CB	5 % Donkey normal serum	
Rabbit IgG-biotin	Goat	Undiluted	SABPO(R) Kit, Nichirei, Tokyo, Japan			Secondary antibody
Rat IgG-biotin	Goat	1:100	BioLegend, San Diego, CA, USA			
Rabbit IgG-Alexa Fluor 488	Donkey	1:500	Thermo Fisher Scientific, Waltham, MA, USA			
Rabbit IgG-Alexa Fluor 546	Donkey	1:500	Thermo Fisher Scientific, Waltham, MA, USA			
Mouse IgG-Alexa Fluor 546	Donkey	1:500	Thermo Fisher Scientific, Waltham, MA, USA			
Rat IgG-Alexa Fluor 488	Donkey	1:500	Thermo Fisher Scientific, Waltham, MA, USA			

CASP3: Caspase 3, BrdU: Bromodeoxyuridine, FOXP3: Forkhead box P3, C3: Complement 3, WT-1: Wilms tumor 1 homolog, CB: 10mM citrate buffer (pH 6.0), 115°C, 15 min. Tris: 20mM tris(hydroxymethyl)aminomethane-HCl (pH 9.0), 115°C, 15 min.

2

3

4 **Supplementary Table S2. Primers used in this study.**

Genes (Accession No.)	Primer Sequence (5'-3') F: Forward, R: Reverse	Product size (bp)
<i>Casp1</i> NM_009807	F: GGTCTTGTGACTTGGAGGACA R: GGGTCACCCTATCAGCAGTG	96
<i>Casp3</i> NM_009810	F: GGATCAAAGCGCAGTGTCTCT R: CCCAGAGTCCACTGACTTGC	145
<i>Casp4</i> NM_007609	F: CGATGTGGTGGTGAAAGAGGA R: CACCAGGAATGTGCTGTCTGA	106
<i>Casp6</i> NM_	F: AGTGTCAGAGCCTGGTTGGA R: TGCAGACAGAGTAGCACATGA	197
<i>Casp7</i> NM_	F: CAACGACATTGACGCTAATCC R: GAGCATGGACACCATAACG	280
<i>Casp8</i> NM_007609	F: CGATGTGGTGGTGAAAGAGGA R: CACCAGGAATGTGCTGTCTGA	106
<i>Casp9</i> NM_001277932	F: GCGACATGATCGAGGATATTCAG R: CAGGAGATGAAGAGAGGAAGGG	118
<i>Casp12</i> NM_009808	F: TAATGCTGACAGCTCCTCATGG R: TCCCTCCTTCTCCATCACTGG	134
<i>Casp14</i> NM_009809	F: TGATCCTCAGCCATTGCAGG R: GGGATCCCTCTTCATGGTGC	163
<i>Dffa</i> NM_001025296	F: TTTGAGGATGGAGCTGTTCGC R: TCCACTATGGTCCCGTCCTC	219
<i>Fas</i> NM_007987	F: CTGCAGACATGCTGTGGATCT R: TATCAGTTTCACGAACCCGCC	132
<i>Bcl2</i> NM_009741	F: GAGTACCTGAACCGGCATCT R: GAGCAGGGTCTTCAGAGACAG	129
<i>Bax</i> NM_007527	F: ATCCAAGACCAGGGTGGCT R: GTGAGGACTCCAGCCACAAA	92
<i>Rock1</i> NM_009071	F: CGGGCAAGAAGGTATCGTCA R: ACCAGGGCATCCAATCCATC	147
<i>Il1b</i> NM_008361	F: TTCCAGGATGAGGACATGAGC R: GACAAACTTCTGCCTGACGAG	111
<i>Tnf</i> NM_013693	F: TCTTCTCATTCTGCTTGTGGC R: CATAGAACTGATGAGAGGGAGGC	119

6 **Supplementary Figure S1. Phenotypes of mucosa-associated lymphoid tissues (MALT) in**  
7 **caspase 3 knockout mice.**

8 For Supplementary figure S1a-c, to examine the proliferation in some mice, bromodeoxyuridine  
9 (BrdU) were intraperitoneal administrated at 100 mg/kg BW at 2 hours before sampling. The  
10 ileum including MALT was fixed with 4 % paraformaldehyde (PFA) at 4 °C overnight. The  
11 nasopharyngeal tissues including the bones were fixed with Bouin's solution for overnight and  
12 immersed in acetone overnight to eliminate lipid. Then, they were immersed in 10% formic acid  
13 for 4 hours to decalcify and washed with water.

14 For Supplementary figure S1, in the immunohistochemistry for BrdU (panels a, a', c, and c')  
15 and forkhead box P3 (FOXP3, panels a'', a''', c'', and c'''), the author selected some lymphoid  
16 clusters from ileum MALT (Peyer's patches) and nasopharynx. Furthermore, the area of lymphoid  
17 clusters and the number of positive cells were quantified by NDP.view2 (Hamamatsu Photonics  
18 Co., Ltd., Hamamatsu, Japan), and the positive cell number to examined area was calculated  
19 (panel b).

20 As a results, BrdU-incorporating cells or FOXP3-positive regulatory T-cells detected in the  
21 periphery of lymph nodes in Peyer's patches are not different between WT (panels a and a'') and  
22 KO mice (panels a' and a'''). Immunohistochemistry using females at 8 months. Bars = 200 μm.  
23 For measurement of the BrdU-incorporating cells or FOXP3-positive regulatory T-cells in lymph  
24 nodes in Peyer's patches using females at 8-12 months, there was no significant difference  
25 between WT and KO mice (panel b). Values are mean ± SE. Mann-Whitney *U*-test. For the  
26 nasopharyngeal tissues, there was no difference of the localization of these positive cells between  
27 WT and KO mice (panel c-c''').

28

29 **Supplementary Figure S2. Glomerular pathology of caspase 3 knockout mice.**

30 Glomerular features in caspase 3 knockout (KO) mice. Periodic acid-Schiff staining using mice

31 at 6 months. There is no clear lesion in both sexes (panels a and a'). Bars = 50  $\mu$ m.