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1 **Desmoplakin (*Dsp*) conditional knockout in NR5A1⁺ somatic cells affects**
2 **germ cell survival in developing mouse gonads**

3

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23

24 **Short title:** *Dsp* involved in gonad development

25 **Keywords:** desmoplakin; cell-cell interactions, cell adhesion; germ cells, testis; testis cords,
26 ovary; ovigerous cords, Sertoli cells, interstitium, follicular cells, gonad development; sexual
27 differentiation; mouse

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29

30 **Abstract**

31 Cell-cell interactions are crucial for morphogenesis and tissue formation. Desmoplakin
32 (encoded by the *Dsp* gene) is a component of desmosomes and anchors the transmembrane
33 adhesion proteins to the cytoskeleton. Its role in gonad development remains vague. To study
34 the role of desmoplakin in gonad development, we used a tissue-specific knockout of the *Dsp*
35 gene in the NR5A1⁺ somatic cells of the gonads. We show here that desmoplakin is necessary
36 for the survival of germ cells in fetal testes and ovaries. The *Dsp* knockout in NR5A1⁺
37 somatic cells in testes decreased the number of germ cells, and thus the size of the testes, but
38 did not affect the Sertoli cells or the structure of testis cords and interstitium. The *Dsp*
39 knockout in NR5A1⁺ somatic cells in ovaries decreased the number of female germ cells and
40 drastically reduced the formation of ovarian follicles. *Dsp* knockout in NR5A1⁺ somatic cells
41 did not affect the sex determination and sexual differentiation of the gonads, as judged from
42 an unchanged expression of genes essential for these processes. We conclude that mediated
43 by desmoplakin cell adhesion between the gonadal cells is necessary for germ cell survival.

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50 **Introduction**

51 The development of gonads is decisive for sexual development and reproduction.
52 Testes and ovaries differentiate from the common progenitor termed the genital ridge (Piprek
53 et al. 2016). During sexual differentiation, the gonadal primordium acquires the sex-specific
54 features and forms either the testis or the ovary (Ungewitter and Yao 2013). In testes, the
55 germ cells become enclosed by differentiating Sertoli cells (pre-Sertoli cells), which adhere to
56 the germ cells and each other. The germ cells surrounded by the Sertoli epithelium built the
57 testis cords (Svingen and Koopman 2013). Sertoli cell epithelium is underlined by the
58 basement membrane. The peritubular myoid cells are located below the Sertoli epithelium and
59 form the outside border of the testis cords (Svingen and Koopman 2013). The interstitial cells,
60 including fetal Leydig cells, which produce androgens (Svingen and Koopman 2013), fill the
61 space between testis cords. In the differentiating ovaries, the ovigerous cords contain germ
62 cells enclosed by somatic pre-follicular cells. In mouse foetus, around the time of birth, the
63 ovigerous cords break down into individual ovarian follicles (Grive and Freiman 2015). The
64 ovigerous cords are much less compact and defined than the testis cords. Differences in male
65 and female gonad tissue structure suggest that the proper intercellular adhesion should be
66 much more important for the development of the testis than the ovaries (Piprek et al. 2017a).

67 In developing testes, strong adhesion between somatic cells facilitates the formation of
68 Sertoli epithelium and the development of solid, elongated testis cords (Piprek et al. 2017a).
69 We showed previously that the variety and the level of expression of the mouse cell adhesion
70 molecules (CAMs) are greater in the male (XY) than in the female (XX) gonad (Piprek et al.
71 2017a). We also showed that the sex-specific cell-cell interactions facilitate the development
72 of *Xenopus laevis* gonads (Piprek et al. 2017b). Although these argue for the importance of
73 CAMs in vertebrate gonad development, our knowledge of specific roles of individual CAMs

74 in gonad development is limited, and a very high number (220) of identified CAMs
75 complicates the studies.

76 Desmoplakin, encoded by the *Dsp* gene, is one of the CAMs showing the sex-specific
77 differences in expression pattern, with a higher expression in the pre-Sertoli cells than in pre-
78 follicular cells (Piprek et al. 2017a). This sex-specific expression pattern suggests
79 desmoplakin involvement in the sexual differentiation of the bipotential gonads into testes or
80 ovaries. However, the role of desmoplakin in gonad development remains unknown.

81 To fill this gap, we studied the role of desmoplakin (*Dsp*) in mouse gonad
82 development. This protein is present in desmosomes and is critical for desmosome assembly,
83 stability, and its interactions with the cytoskeleton (Gallicano et al. 1998; Bornslaeger et al.
84 1996; Kowalczyk and Green 2013). Mutations in desmoplakin lead, directly or indirectly, to
85 various diseases, such as cardiomyopathies, skin conditions such as keratoderma, and
86 oropharyngeal and breast cancers (Norgett et al. 2000; Pang et al. 2004; Papagerakis et al.
87 2009). In mice, conventional whole-body knockout of desmoplakin eliminates the function of
88 desmosomes, disrupts blastomere adhesion, and leads to embryo lethality before stage E6.5
89 (Gallicano et al. 1998).

90 In this study, we used conditional knockout (cre-loxP system) of desmoplakin to
91 overcome embryo lethality. Because we were especially interested in the role of desmoplakin
92 in the somatic-germ cell interactions during development, we removed the exon 2 of the *Dsp*
93 gene, specifically in the somatic cells of developing gonads. From our previous studies on the
94 knockout of E- and N-cadherin adhesion molecules, we knew that the absence of these CAMs
95 in somatic cells of the gonads results in alteration of germ cell development (Piprek et al.
96 2019a,2019b). Here we crossed a mouse strain expressing the *Cre* recombinase under *Nr5a1*
97 promoter with a strain containing *loxP* sequences flanking exon 2 in the *Dsp* gene. The
98 resulting deletion of exon 2 made the desmoplakin nonfunctional (Vasioukhin et al. 2001) in

99 NR5A1-positive (NR5A1⁺) cells. The *Nr5a1* (Nuclear Receptor Subfamily 5 Group A
100 Member 1, also known as *Sfl* - Steroidogenic Factor 1) is expressed from stage E10.2 in the
101 coelomic epithelium covering the genital ridges (Ikeda et al. 1994; Hu et al. 2013). The
102 NR5A1⁺ cells of the coelomic epithelium give rise to Sertoli cells and a subpopulation of
103 interstitial cells in the testes, and follicular cells and a subpopulation of theca cells in the
104 ovaries (Albrecht and Eicher 2001; DeFalco et al. 2011; Liu et al. 2015). Thus, the knockout
105 we have performed eliminated desmoplakin expression only from those somatic cells, which
106 are known to be critical for sex determination and gonad development. Such NR5A1⁺ ~~Sfl~~⁺
107 cell-specific knockout was important because we expected that eliminating desmoplakin from
108 all somatic cells of gonads would completely halt gonad development. We studied the gonad
109 phenotypes by light microscopy, immunohistochemistry and analyzed the expression of
110 selected genes by RT-qPCR.

111

112 **Materials and methods**

113 *Animals*

114 The study was approved by the First Local Commission for Ethics in Experiments on
115 Animals. The animals were bred and housed in the Animal Facility at the Jagiellonian
116 University (Krakow, Poland). Two transgenic mouse lines used in this study were purchased
117 from The Jackson Laboratory (ME, USA).

118 The mouse strain Tg(Nr5a1-cre)7Low1/J (Dhillon et al. 2006) was used for the *Cre*
119 recombinase expression in NR5A1⁺ somatic cells of the gonads. The *Dsp* gene coding for
120 desmoplakin was deleted in B6.129-*Dsp*^{tm1Efu}/J mouse strain (Vasioukhin et al. 2001). *Cre*⁺,
121 *loxP*^{fl/fl}, and *Cre*⁻, *loxP*^{+/+} specimens were used as a negative control. The numbers of studied
122 individuals are presented in Table 1.

123 Timed mating was performed by placing a single male with two females overnight.
124 The following morning, females were checked for the presence of the vaginal plug, and the
125 pregnancies were designated as E0.5 (embryonic day). Females were sacrificed by spinal cord
126 dislocation at embryonic days: E10.5, 11.5, 12.5, 13.5, 14.5, 16.5, 18.5, and the newborns
127 were sacrificed at 1 and 2 *dpp* (days *postpartum*).

128

129 ***Genotyping***

130 The sex of all studied individuals was identified by the genotyping using primers for
131 *Sly* (Y chromosome) and *Xlr* (X chromosome) (McFarlane et al. 2013). Primers used to
132 genotype *Cre*⁺, and *loxP*⁺ specimens are listed in Table 2. We used a standard PCR protocol
133 for genotyping.

134

135 ***RNA isolation from gonads and Real-Time Quantitative PCR***

136 Gonads from mouse fetuses and newborns were pooled according to the sex and
137 developmental stage. Total RNA was isolated using Trizol and further purified with RNeasy
138 Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Total RNA in
139 RNase-free water was stored at -80°C and used for multigene qPCR analysis. 50 ng RNA of
140 each sample was reverse-transcribed into cDNA using random primers and SuperScript III
141 Reverse Transcriptase (Invitrogen, 18080044) according to the manufacturer's instructions. A
142 list of primers is shown in Table 2. RT-qPCR was performed in 5µl reactions using SYBR
143 Green Master Mix (Life Technologies, 4312704) on a 7500 Fast Real-Time PCR System
144 (Applied Biosystems) with universal cycling parameters and analyzed as previously described
145 (Svingen et al. 2009). *Actb* was used as a reference gene. Statistical analysis was performed
146 using the nonparametric ANOVA Kruskal-Wallis test followed by Tukey's test. Statistica 7.0
147 software was used for the analyses.

148 ***Histology and Immunohistochemistry***

149 Dissected gonads were fixed in Bouin's solution, dehydrated, and embedded in
150 paraffin (Paraplast, Sigma, P3683). Histological staining was performed according to
151 Debreuill's trichromatic method as previously described (Kiernan 1990; Piprek et al. 2017b).
152 For immunochemistry, heat-induced epitope retrieval was conducted in sodium citrate buffer
153 (10 mM sodium citrate, 0.05% Tween-20, pH 6) at 95°C for 20 minutes. Subsequently, the
154 sections were blocked with 3% H₂O₂ and 10% goat serum (Sigma, G9023). Sections were
155 incubated with the primary antibodies (rabbit polyclonal: anti-AMH, Santa Cruz
156 Biotechnology, sc-166752; anti-collagen I, Abcam, ab34710; anti-GCNA1, Abcam, ab82527,
157 anti-CYP17A1, Abcam, ab125022; anti-PCNA, Abcam, ab18197; anti-cleaved caspase 3,
158 Assay BioTech, L0104; anti-Cre recombinase, Abcam, ab190177; and mouse monoclonal
159 anti-desmoplakin, Santa Cruz Biotechnology, sc-390975) at 4°C overnight, and the signal was
160 detected using UltraVision Quanto Detection System (Thermo Fisher, TL-125-QHD).
161 Mayer's hematoxylin was used as a counterstain. Sections were examined under the Nikon
162 Eclipse E600 microscope.

163

164 ***Germ cell quantification***

165 The sections of the gonads from the animals sacrificed at 2 *dpp* were stained with
166 Trichrome. The number of germ cells was calculated in the 10,000 μm^2 area in 5 cross-
167 sections from each gonad using the ImageJ software. The diameter of testes, testis cords, and
168 ovaries was measured in the five widest cross-sections. Average values and standard
169 deviation were calculated using Microsoft Excel software.

170

171

172

173 **Results**

174 *The effectiveness of genetic knockout*

175 To assess the effectiveness of the knockout, first, we checked the expression of *Cre*
176 recombinase using RT-qPCR and immunohistochemistry. There was a high expression of *Cre*
177 gene under the control of the *Nr5a1* promoter in fetal XY and XX gonads from E11.5 (Fig.
178 1A, D). In both sexes, the expression of *Cre* mRNA declined from E16.5 onward (Fig. 1A,
179 D). The control testes did not express the CRE protein (Fig. 1B). In the knockout testes, the
180 CRE protein was expressed in all Sertoli cells and some, but not all interstitial cells (Fig. 1C,
181 see the dark brown staining labeled with arrows and arrowheads). In control ovaries, the CRE
182 was not expressed (Fig. 1E). In the knockout ovaries the CRE protein was expressed in the
183 somatic cells (Fig. 1F, arrows).

184 The declining expression of *Dsp* from E11.5 onward in the gonads of both sexes (Fig.
185 1G, J) proved the effectiveness of the knockout. Because the desmoplakin knockout was
186 induced only in a subpopulation of the gonadal cells, low desmoplakin expression was still
187 detectable in the gonad. Staining with antibodies against the fragment encoded by the deleted
188 exon 2 of the *Dsp* gene showed a clear difference in the intensity of desmoplakin
189 immunostaining between the control and the knockout testes and ovaries (Fig. 1H and 1K,
190 and compare to Fig. 1I and 1L, respectively), which indicated successful knockout of *Dsp*
191 expression in the somatic cells of developing male and female gonads.

192

193 *The effect of Dsp knockout in NR5A1⁺ cells on the number of germ cells*

194 The testes of knockout males were smaller than the control testes. The apparent
195 difference in size became visible starting from E16.5 (Fig. 2A). Histological analysis and
196 immunolocalization of GCNA1 (germ cell marker) showed that the decrease in the size of
197 testes correlated with the reduction of germ cell number (Fig. 2B-D). Consistent with the

198 reduction in germ cell number observed in ovaries and testes from around E16.5, the
199 expression of the germ cell marker *Mvh* (mouse vasa homolog) was also reduced in E16.5
200 testes and ovaries (Fig. 2G).

201 The ovaries of the knockout females had fewer germ cells, were smaller than the
202 control ovaries (Fig. 2A), and the difference in size was apparent from E16.5. The number of
203 cells positive for GCNA1 was lower in the knockout than in control (Fig. 2B, E, F). The level
204 of *Mvh* expression was lower than in control (Fig. 2G; XX gonads are shown in the middle).

205 To establish if the germ cell loss resulted from a decreased proliferation or increased
206 apoptotic cell death, we performed immunostaining with the antibody against proliferation
207 marker PCNA and the apoptotic marker caspase 3 (Fig. 3). We did not detect any changes in
208 the PCNA expression in fetal testes or ovaries (compare Fig. 3A with 3B, and 3E with 3F;
209 Fig. 3I shows a graphical presentation of the expression in time). However, caspase 3 had
210 ~~higher expression~~ higher expression (stronger immunostaining) in the fetal knockout testes
211 (compare Fig. 3C with 3D, apoptotic cells are indicated by the arrowheads, Fig. 3J shows a
212 graphical representation of the expression in time), and ovaries (compare Fig. 3G with 3H,
213 graphical presentation in Fig. 3J). The location of the apoptotic cells and their large nuclei
214 suggest that they are germ cells. These data strongly suggest that the loss of germ cells in
215 testes with the *Dsp* knockout in NR5A1⁺ cells resulted from apoptosis triggered by the
216 absence/diminution of desmoplakin in gonadal somatic cells.

217

218 ***The effect of Dsp knockout in NR5A1⁺ cells on testes development***

219 Although the size of control and knockout testes was different, the testis structure
220 remained unchanged; both contained many testis cords (ts, encircled) divided by interstitium
221 (i) (Fig. 4A-D).

222 We found that the developing *Dsp* knockout testes had typical testis cords (Fig. 4B, D,
223 F, H, J) like those in control (Fig. 4A, C, E, G, I). Also, the basement membrane and the
224 peritubular myoid cells (PMCs) located around the cords had normal morphology (Fig. 4C, D;
225 basement membrane (arrow), peritubular myoid cells (pmc)). The AMH (anti-Müllerian
226 hormone, which is a specific marker of Sertoli cells) immunostaining showed that in the
227 control testis, the positively stained Sertoli cells enclosed numerous germ cells (Fig. 4E, G;
228 germ cells (g)). The knockout testes lacked the germ cells, and their testis cords were filled
229 with the AMH-positive Sertoli cells (Fig. 4F, H). The diameter of testis cords was
230 significantly lower in the knockout testes (mean diameter $41.82 \mu\text{m} \pm \text{SD } 6.51$ at E18.5)
231 than in control ($49.10 \mu\text{m} \pm \text{SD } 4.80$ at E18.5), an effect that is likely to be caused by the
232 loss of relatively voluminous germ cells. Collagen I immunostaining of control testes (Fig. 4I)
233 and knockout testes (Fig. 4J) showed that the sterile cords in the knockout testes were
234 enclosed by typically looking basement membranes (indicated by arrows in Fig. 4I and J).
235 This indicates that the knockout of desmoplakin under *Nr5a1* promoter in testis cells has not
236 affected the integrity of the somatic component of the gonad. In the control testes, the space
237 between the testis cords is occupied by the interstitium (i) (Figs 4C, E, G, I). We did not
238 observe any structural changes in the interstitium of the knockout testes (Fig. 4D, F, H, J).

239

240 ***The effect of *Dsp* knockout in NR5A1⁺ cells on ovarian development***

241 The knockout ovaries contained very few germ cells located in the ovarian surface
242 epithelium, while the control ovaries were filled with many germ cells (compare Fig. 5A, C
243 with Fig. 5B, D). The ovigerous cords were present both in the control and knockout ovaries
244 (compare Fig. 5A, C with Fig. 5B, D; ovigerous cords are marked by a dotted line). The
245 ovigerous cords of the control ovaries consist of the elongated groups of germ cells
246 surrounded by the somatic cells. The ovigerous cords of the knockout ovaries consist of

247 somatic cells only (Fig. 5B, D). This indicates that the knockout of desmoplakin under *Nr5a1*
248 promoter in ovarian cells did not affect the somatic cell adhesion and aggregation necessary
249 for the formation of ovigerous cords. The control ovaries contained the ovarian follicles
250 (encircled by a solid line in Fig.5A) which consisted of a single germ cell enclosed by a
251 monolayer of the somatic cells. However, in the knockout ovary, the ovarian follicles were
252 absent (Fig. 5D). As we have previously suggested (Piprek et al. 2019a, 2019b), this may
253 indicate that germ cells are critical for forming ovarian follicles. Alternatively, the loss of *Dsp*
254 may interfere with the breakdown of ovarian cysts and/or the formation of ovarian follicles
255 resulting in the loss of germ cells.

256

257 ***Dsp* knockout in NR5A1⁺ cells does not affect sex determination**

258 The expression of three selected Sertoli cell markers and genes involved in male sex
259 determination (*Sox9*, *Amh*, *Dhh*) studied by RT-qPCR were unaltered in the knockout testes
260 (Fig. 6A). Thus, the knockout of desmoplakin performed in this study did not affect the
261 pathways of Sertoli cell differentiation and male sex determination. Accordingly, the analysis
262 performed by RT-qPCR in control and NR5A1⁺ cells KO ovaries showed no change in the
263 expression of three selected ovarian-specific genes: *Rspo1*, *Wnt4*, *Foxl2* (Fig. 6B). All these
264 data indicate that desmoplakin deletion in NR5A1⁺ cells does not affect male or female sex
265 determination.

266 The expression of the steroidogenic enzyme *Cyp11a1*, which is a specific marker of
267 steroidogenic Leydig cells, was comparable in the knockout and control testes (Fig. 6A).

268

269 **Discussion**

270 The role of desmoplakin in gonad development was unknown before our study. The
271 only information available was that the desmosomes are important for spermatogenesis in

272 adult testes (Mruk and Cheng 2011). In our study, the use of CRE recombinase under the
273 control of *Nr5a1* promoter allowed us to delete desmoplakin in the descendants of NR5A1⁺
274 coelomic epithelium, *i.e.*, Sertoli cells and a subpopulation of interstitial cells in the testes,
275 and the follicular cells and stromal cells in the ovaries (Ikeda et al. 1994; Albrecht and Eicher
276 2001; DeFalco et al. 2011; Hu et al. 2013, reviewed in Pipek et al. 2016). We provided
277 strong evidence that desmoplakin in the somatic cells of developing gonads is critical for
278 germ cell survival and maintenance in male and female gonads. The developing knockout
279 testes and ovaries were smaller than the control and had fewer germ cells. In both sexes, the
280 germ cell loss occurred at embryonic day E16.5. Previously, we showed that the deletion of
281 N-cadherin or E-cadherin in the gonadal somatic cells caused germ cell loss (Pipek et al.
282 2019a, b). It is unclear why germ cell loss occurs during this particular period of
283 embryo/gonad development. The decreased expression of *Dsp* in gonadal somatic cells was
284 apparent from stage E11.5 onward, so it is possible that it takes several days (between stage
285 E11.5 and E16.5) before the loss of desmoplakin in the somatic cells affects the germ cells.
286 Alternatively, the germ cells could be especially susceptible to desmoplakin loss. In testes,
287 most germ cells enter mitotic arrest between E12.5 and E14.5, and by E15.5 they are all in the
288 mitotic arrest (Western et al. 2008). In the ovaries, at stage E17.5 some of the germ cells enter
289 the diplotene stage of meiosis, and the germ cysts break down into ovarian follicles (Borum
290 1961, Pepling and Spradling 2001, Pepling et al. 2010). The knockout of desmoplakin may
291 possibly loosen cell adhesion, which in turn may induce pro-apoptotic signaling, as it occurs
292 in the case of carcinogenesis. Although the molecular mechanism of the germ cell loss is
293 unclear, the E-cadherin-mediated contacts prevent apoptosis in rat granulosa cells, and the
294 deletion of E-cadherin activates caspase 3-dependent apoptosis by inhibiting Akt kinase
295 (protein kinase B) (Peluso et al. 2001). N-cadherin inhibits apoptosis by mediating Akt
296 signaling and PI3K (phosphatidylinositol-3 kinase) pathway (Tran et al. 2002). Its deletion

297 causes germ cell loss through the down regulation of these pathways (Piprek et al. 2019a, b).
298 A mechanism by which a cell adhesion molecule can mediate cell proliferation and apoptosis
299 may be cell type-specific. Previous studies showed that the knockdown of desmoplakin in
300 human HaCaT keratinocytes increased cell proliferation and elevated phospho-Akt and
301 phospho-ERK1/2 levels (Wan et al. 2007). The upregulation of desmoplakin in non-small cell
302 lung cancer (NSCLC) inhibited proliferation and increased sensitivity to apoptotic signals
303 (Yang et al. 2012).

304 We showed here that in the absence of desmoplakin, somatic components of gonads in
305 both sexes developed normally. Similarly, the knockout of the E-cadherin did not cause
306 changes in the somatic component of the gonad, only the loss of germ cells (Piprek et al.
307 2019a). However, the knockout of N-cadherin led not only to the loss of germ cells but also to
308 the disruption of testis cords and loss of fetal Leydig cells (Piprek et al. 2019b). Thus, N-
309 cadherin, but not E-cadherin and desmoplakin, is critical for the formation of somatic
310 components of the gonad.

311 The absence of ovarian follicles in the knockout gonads was the only visible sign of
312 the change in the somatic part of the gonad. The same effect was also observed in N- and E-
313 cadherin knockouts (Piprek et al. 2019a, b). Hypothetically the disruption in the ovarian
314 follicle formation can be an effect of the lack of germ cells, or alternatively, granulosa cells
315 may require desmoplakin for cell adhesion to assemble ovarian follicles. The failure of the
316 germ cyst break down into the ovarian follicles may possibly underlie the massive germ cells
317 death observed under *Dsp* knockout conditions.

318 We also studied the expression of male and female sex-determining genes. We showed
319 that the knockout of desmoplakin in NR5A1⁺ cells, similarly to N- and E-cadherin, did not

320 affect the expression of these genes (Piprek et al. 2019a, b). Thus, it seems that cell adhesion
321 mediated by these proteins is not crucial for sex determination processes.

322 Further studies are necessary to uncover the potential interactions between Akt and
323 PI3K signaling pathways, cell adhesion, apoptosis, and the germ cells of developing gonads.

324

325 **Declaration of interest**

326 The authors do not have conflict of interest.

327

328 **Author contributions**

329 Conceptualization: R.P.P.; Methodology: R.P.P.; Validation: R.P.P., I.R.; Formal analysis:
330 R.P.P., I.R.; Investigation: R.P.P., I.R.; Resources: R.P.P.; Data curation: R.P.P.; Writing –
331 original draft: R.P.P., M.K., J.Z.K.; Writing – review & editing: R.P.P., M.K., J.Z.K., RZ;
332 Supervision: R.P.P.; Project administration: R.P.P.; Funding acquisition: R.P.P.

333

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337

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472

473 **Figure legends**

474 **Fig. 1. Cre and Dsp expression.** **A.** The expression of *Cre* recombinase in XY gonads is high
475 from E11.5 onward. **B, C.** Immunostaining of CRE recombinase at E18.5 shows a lack of
476 CRE recombinase in the control testis (*Nr5a1-Cre⁻Dsp^{+/+}*). The knockout testis (*Nr5a1-*
477 *Cre⁺Dsp^{fl/fl}*) express CRE in Sertoli cells (arrowheads) and interstitial cells (arrows). **D.** The
478 expression of *Cre* recombinase in XX gonads is high from E11.5 onward. **E, F.**

479 Immunostaining of CRE recombinase at E18.5. The control ovary (*Nr5a1-Cre⁻Dsp^{+/+}*) does
480 not express Cre. The somatic cells (arrows) of the knockout (*Nr5a1-Cre⁺Dsp^{fl/fl}*) express Cre.

481 **G.** The expression of *Dsp* is significantly lower (from E11.5 onward) in the knockout XY

482 gonads than in control. **H, I.** Immunostaining of desmoplakin in the testes at E18.5.

483 Desmoplakin is present in the control (**H**) but not in the knockout testis (**I**). **J.** The expression
484 of *Dsp* is significantly lower (from E11.5 onward) in the knockout XX gonads than in control.

485 **K, L.** Immunostaining of desmoplakin in the ovaries at E18.5. Desmoplakin is present in the

486 control ovary (**K**) but not in the knockout ovary (**L**). * $P < 0.05$, n - number of individuals

487 tested, bars indicate standard deviation. Scale bar: 25 μ m.

488

489 **Fig. 2. Gonad size and the number of germ cells.** **A.** The knockout XY and XX gonads are
490 smaller than the control starting from E16.5 onward. **B.** Germ cell number in the knockout
491 XY and XX gonads is lower than the control starting from E16.5 onward. **C, D.**

492 Immunostaining of GCNA1 (germ cell marker) shows many germ cells in the control testis

493 (C) and only a few germ cells in the knockout testis (D). E, F. Immunostaining of GCNA1
494 shows many germ cells in the control ovaries and only a few germ cells (arrowhead) in the
495 surface epithelium of the knockout ovary. G. Expression of *Mvh* (germ cell marker) at E18.5
496 in both XY and XX gonads shows a significant decrease in the knockout (KO) compared to
497 the control (C) in both sexes. * $P < 0.05$, n - number of individuals tested, bars indicate
498 standard deviation. Scale bar: 25 μm .

499

500 **Fig. 3. Proliferation (PCNA immunostaining) and apoptosis (caspase 3 immunostaining)**

501 **in desmoplakin knockout testes and ovaries.** The similar intensity of PCNA staining in
502 control (A) and knockout testis (B) indicates a similar cell proliferation rate at E18.5.
503 Apoptotic (caspase 3-positive) cells (arrowheads) are present within the testis cords of the
504 knockout testis (D). In the control testes, the apoptosis is low (C). The knockout and control
505 ovaries have a similar intensity of PCNA signal (E, F). The knockout ovaries show a strong
506 signal of caspase 3 immunostaining, indicating enhanced apoptosis (H) comparing to the
507 control (G). I. A number of PCNA⁺ cells in male and female gonads. There are no significant
508 differences between control and knockout gonads. J. A number of caspase3⁺ cells in male and
509 female gonads. There are significant differences between control and knockout gonads
510 starting from E16.5. * $P < 0.05$, n - number of individuals tested, bars indicate standard
511 deviation. Scale bar: 25 μm .

512

513 **Fig. 4. Histology of the testes in control and desmoplakin knockout mice. A-D.**

514 Histological staining shows a similar gross morphology of control (*Nr5a1-cre⁺Dsp^{+/+}*) and
515 knockout testes (*Nr5a1-cre⁺Dsp^{fl/fl}*) at stage E18.5. However, in the knockout testis, testis
516 cords (ts) contain only a few germ cells (g) enclosed by Sertoli cells (Sc), while in control,
517 testis cords (ts) have many germ cells (g). In the knockout and control testes, the Sertoli cells

518 are enclosed by the basement membrane (arrows) and peritubular myoid cells (pmc). **E-F.**
519 AMH immunostaining shows Sertoli cells in control (**E, G**) and knockout testes (**F, H**). In the
520 knockout testes, the Sertoli cells fill the whole testis cords (ts). **I, J.** Collagen I
521 immunostaining shows the presence of continuous basement membranes (arrows) enclosing
522 the testis cords (ts) in the control and knockout testes. Scale bar: A, B, E, F: 100 μm ; C, D, G-
523 J: 25 μm .

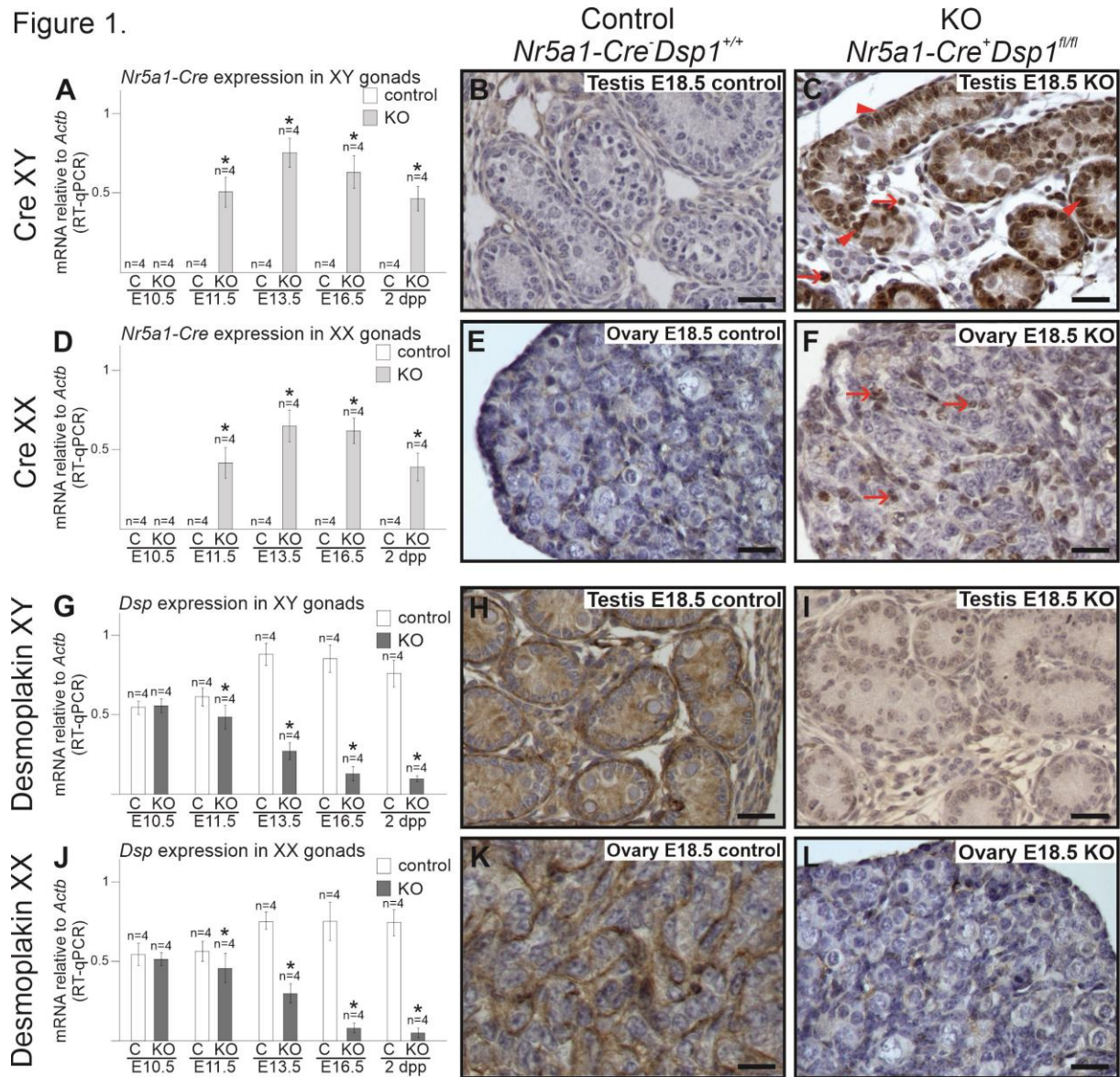
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525 **Fig. 5. Histology of the testes in control and desmoplakin knockout mice.** **A, C)** Histology
526 of control ovary (*Nr5a1-Cre⁺Dsp^{+/+}*) at E18.5 show many oocytes (arrowheads) surrounded
527 by the follicular cells (arrow) in the ovarian cortex. The ovigerous cords are encircled by
528 dotted lines. Sporadically singular oocytes enclosed by follicular cells are present, indicating
529 the formation of the ovarian follicles (encircled by a solid line). **B, D)** Histology of knockout
530 ovary (*Nr5a1-Cre⁺Dsp^{fl/fl}*) shows the loss of oocytes. The sterile ovigerous cords (dotted line)
531 are filled with somatic cells. No ovarian follicles are present. Scale bar: A, B: 100 μm ; C, D:
532 25 μm .

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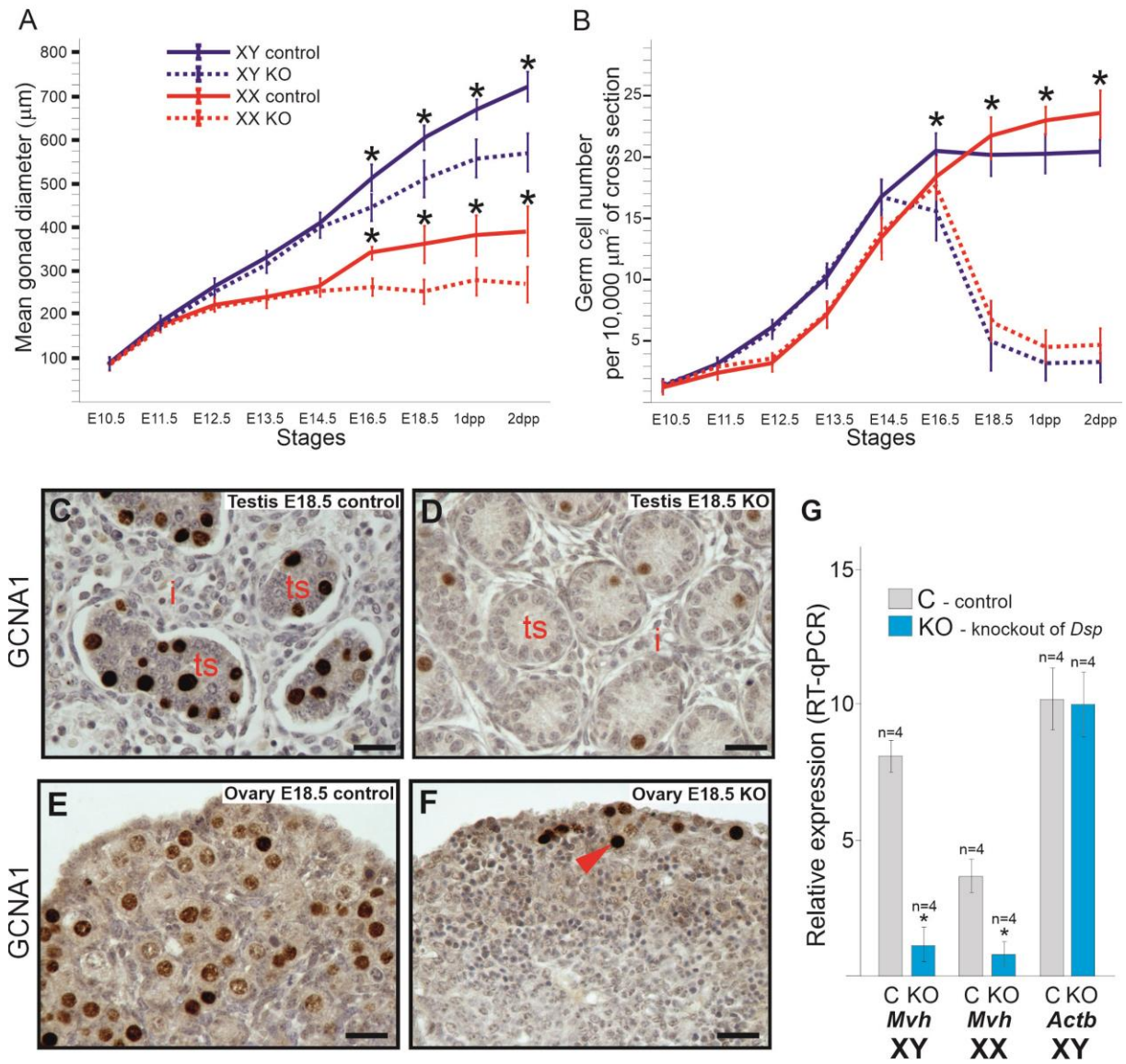
534 **Fig. 6. Gene expression in control (C) and desmoplakin knockout (KO) gonads at E18.5.**
535 **A.** In the XY gonads, the expression of *Amh*, *Sox9*, *Dhh* (Sertoli cell maker and genes of male
536 sex determination), and *Cyp11a1* (a marker of fetal Leydig cell and steroidogenesis) is similar
537 to the control. **B.** In the XX gonads, the expression of *Rspo1*, *Wnt4*, *Foxl2* (makers of pre-
538 follicular cells and female sex determination), and *Cyp11a1* is similar in the knockout ovaries
539 and the control. * $P < 0.05$, n - number of individuals tested, deviation bars indicate standard
540 deviation.

Figure 1.



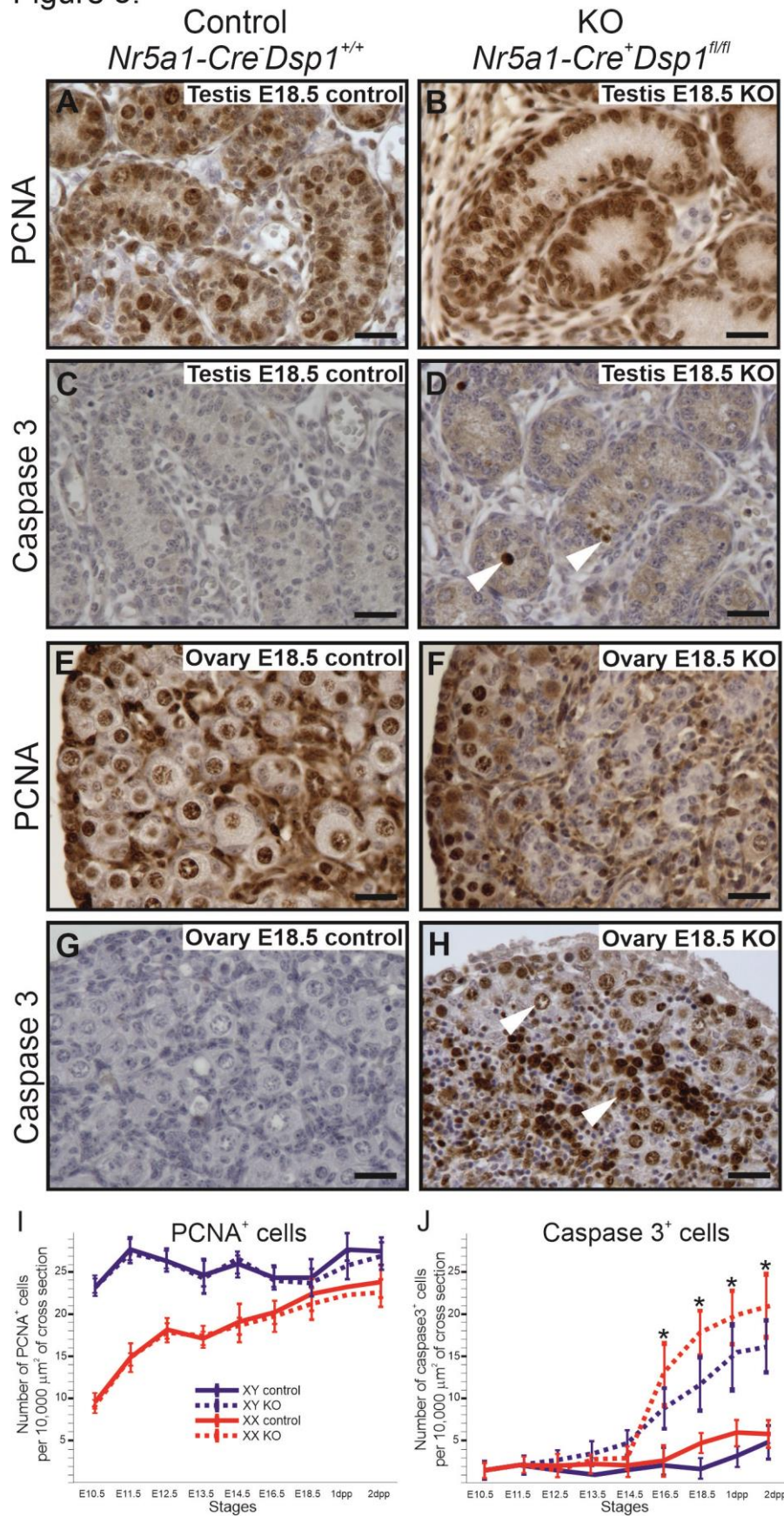
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Figure 2.



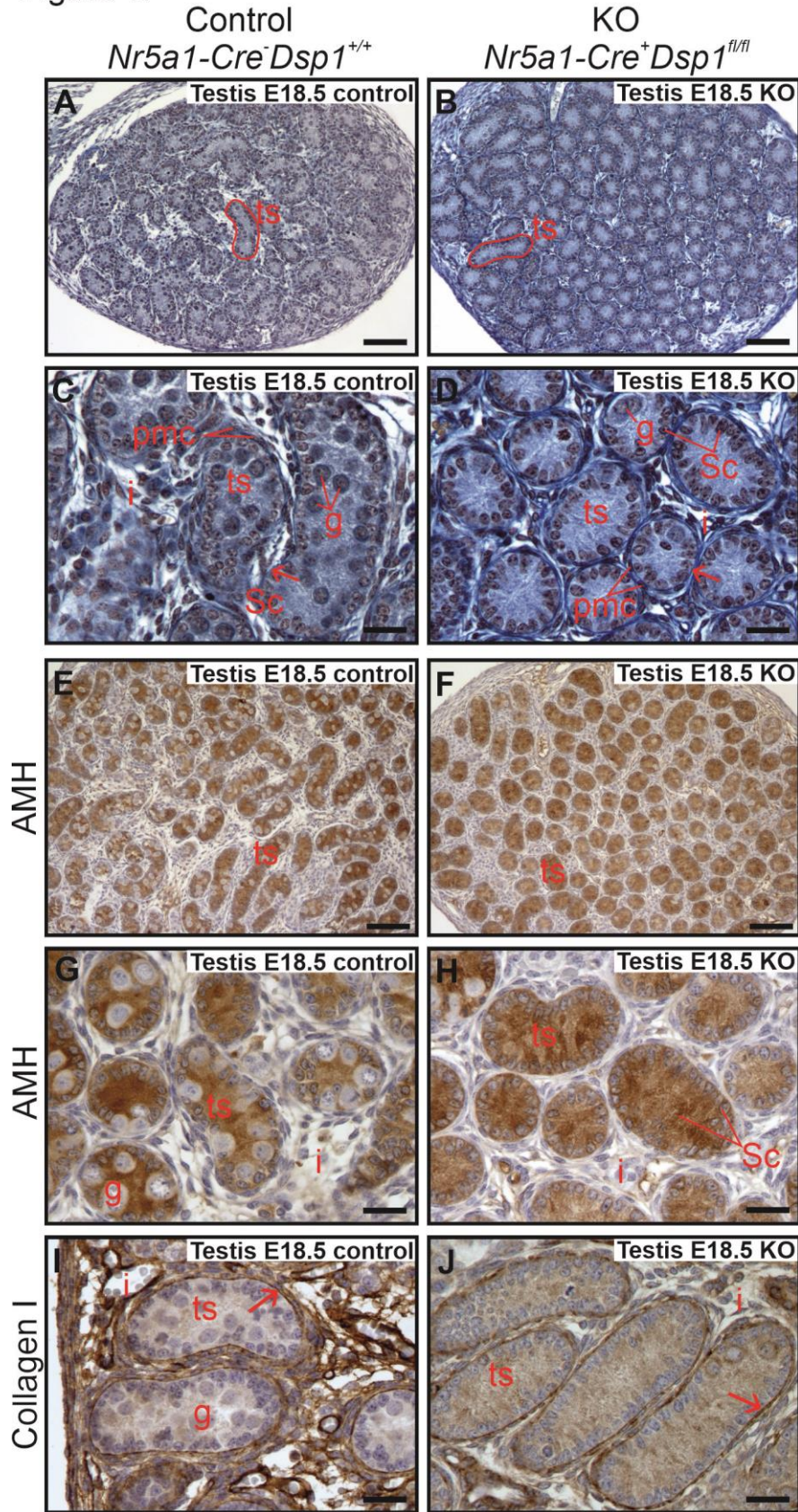
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Figure 3.



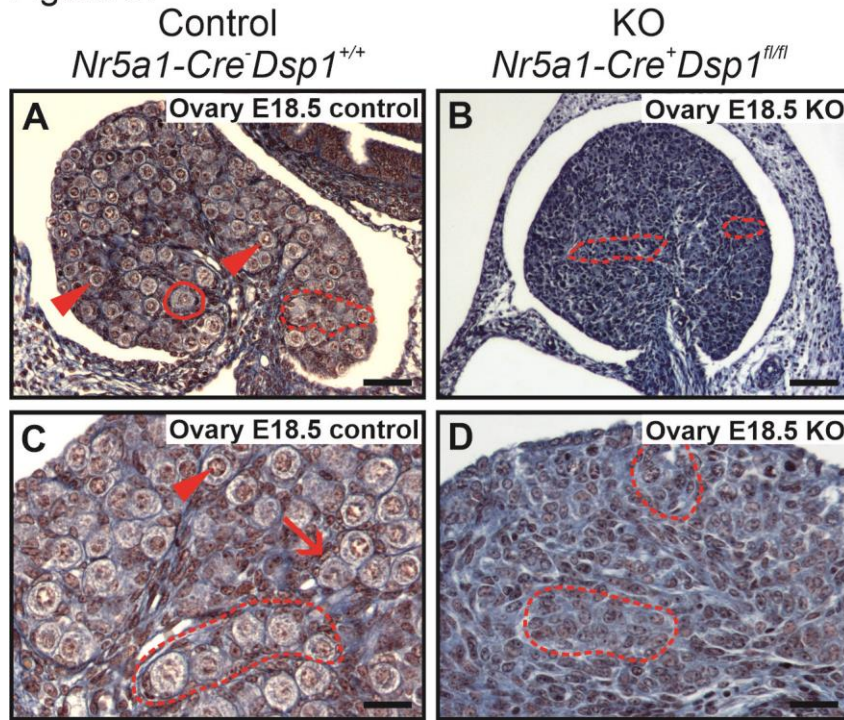
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Figure 4.



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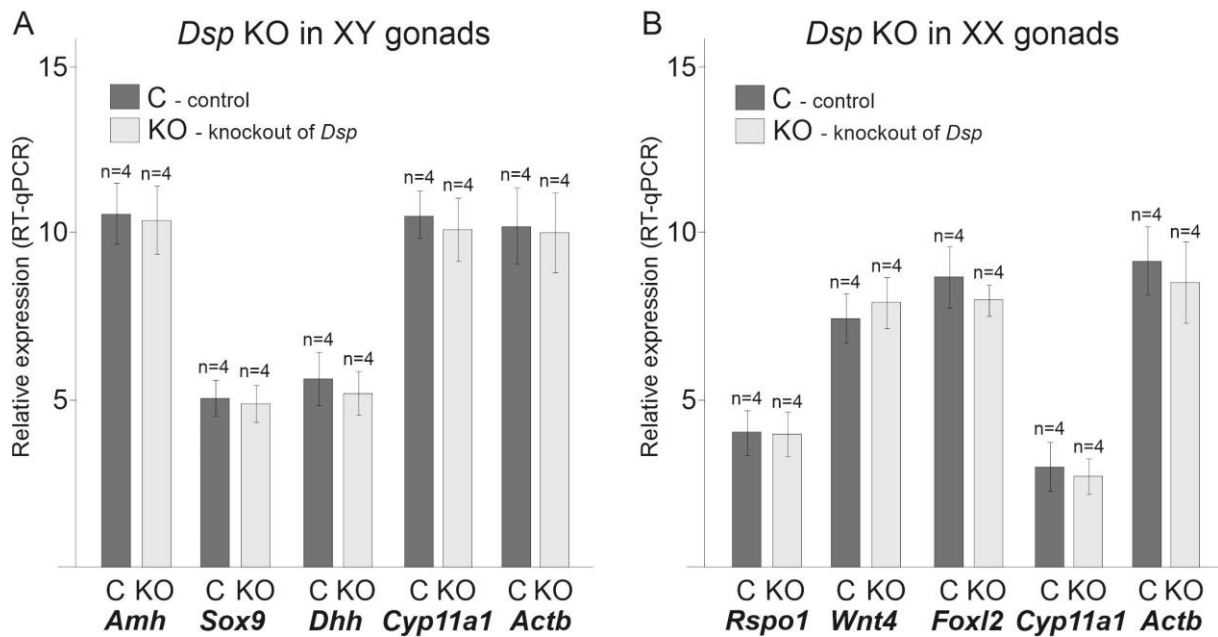
Figure 5.



545

546

Figure 6.



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Table 1. Number of specimens tested.

Genotype	E10.5		E11.5		E12.5		E13.5		E14.5		E16.5		E18.5		1dpp		2dpp		Sum
	XX	XY	XX	XY	XX	XY	XX	XY	XX	XY	XX	XY	XX	XY	XX	XY	XX	XY	
<i>Nr5a1-Cre⁻</i> <i>Dsp^{+/+}</i> control	8	8	8	8	8	8	8	8	10	10	8	8	10	10	8	8	8	8	152
<i>Nr5a1-Cre⁺</i> <i>Dsp^{fl/fl}</i> knockout	8	8	8	8	8	8	8	8	10	10	8	8	10	10	8	8	8	8	152

Table 2. Primers used for genotyping and RT-qPCR.

Gene	Primers
Primers used for genotyping	
<i>SX</i> (sex genotyping)	F: GATGATTTGAGTGGAAATGTGAGGTA R: CTTATGTTTATAGGCATGCACCATGTA
<i>Nr5a1-Cre</i>	Transgene F: CTGAGCTGCAGCGCAGGGACAT Transgene R: TGCGAACCTCATCACTCGTTGCAT Control F: CAAATGTTGCTTGTCTGGTG Control R: GTCAGTCGAGTGCACAGTTT
<i>Dsp-loxP</i>	F: GTTGGGCCTCTCGAATCAT R: TCTTTGTCTGTTGCCATGTGA
Primers used for RT-qPCR	
<i>Actb</i>	F: CATGTACGTTGCTATCCAGGC R: CTCCTTAATGTCACGCACGAT
<i>Amh</i>	F: TCAACCAAGCAGAGAAGGTG R: AGTCATCCGCGTGAAACAG
<i>Cyp11a1</i>	F: GTGAATGACCTGGTGCTTGGT R: TCGACCCATGGCAAAGCTA
<i>Dhh</i>	F: TGATGACCGAGCGTTGTAAG R: GCCAGCAACCCATACTTGTT
<i>Foxl2</i>	F: GCTACCCCGAGCCCGAAGAC R: GTGTTGTCCCGCCTCCCTTG
<i>Mvh</i>	F: GAGATTGCCTTCAGTACCTATGTG R: GTGCTTGCCCTGGTAATTCT
<i>Rspo1</i>	F: TGTGAAATGAGCGAGTGGTCC R: TCTCCAGATGCTCCAGTTCT
<i>Sox9</i>	F: GTGCAAGCTGGCAAAGTTGA R: TGCTCAGTTCACCGATGTCC
<i>Wnt4</i>	F: TGTACCTGGCCAAGCTGTCAT R: TCCGGTCACAGCCACACTT