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Desmoplakin (*Dsp)* conditional knockout in NR5A1^{+} somatic cells affects germ cell survival in developing mouse gonads

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Abstract

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

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Introduction

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

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

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

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

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

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

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NR5A1-positive (NR5A1^{+}) cells. The *Nr5a1* (Nuclear Receptor Subfamily 5 Group A Member 1, also known as *Sf1* - Steroidogenic Factor 1) is expressed from stage E10.2 in the coelomic epithelium covering the genital ridges (Ikeda et al. 1994; Hu et al. 2013). The NR5A1^{+} cells of the coelomic epithelium give rise to Sertoli cells and a subpopulation of interstitial cells in the testes, and follicular cells and a subpopulation of theca cells in the ovaries (Albrecht and Eicher 2001; DeFalco et al. 2011; Liu et al. 2015). Thus, the knockout we have performed eliminated desmoplakin expression only from those somatic cells, which are known to be critical for sex determination and gonad development. Such NR5A1^{+} ~~Sf1^{+}~~ cell-specific knockout was important because we expected that eliminating desmoplakin from all somatic cells of gonads would completely halt gonad development. We studied the gonad phenotypes by light microscopy, immunohistochemistry and analyzed the expression of selected genes by RT-qPCR.

Materials and methods

Animals

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

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

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

Genotyping

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

RNA isolation from gonads and Real-Time Quantitative PCR

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

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Histology and Immunohistochemistry

Dissected gonads were fixed in Bouin's solution, dehydrated, and embedded in paraffin (Paraplast, Sigma, P3683). Histological staining was performed according to Debreuill's trichromatic method as previously described (Kiernan 1990; Piprek et al. 2017b). For immunochemistry, heat-induced epitope retrieval was conducted in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6) at 95°C for 20 minutes. Subsequently, the sections were blocked with 3% H_2O_2 and 10% goat serum (Sigma, G9023). Sections were incubated with the primary antibodies (rabbit polyclonal: anti-AMH, Santa Cruz Biotechnology, sc-166752; anti-collagen I, Abcam, ab34710; anti-GCNA1, Abcam, ab82527, anti-CYP17A1, Abcam, ab125022; anti-PCNA, Abcam, ab18197; anti-cleaved caspase 3, Assay BioTech, L0104; anti-Cre recombinase, Abcam, ab190177; and mouse monoclonal anti-desmoplakin, Santa Cruz Biotechnology, sc-390975) at 4°C overnight, and the signal was detected using UltraVision Quanto Detection System (Thermo Fisher, TL-125-QHD). Mayer's hematoxylin was used as a counterstain. Sections were examined under the Nikon Eclipse E600 microscope.

Germ cell quantification

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

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Results

The effectiveness of genetic knockout

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

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

The effect of Dsp knockout in NR5A1^{+} cells on the number of germ cells

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

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

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

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

The effect of Dsp knockout in NR5A1^{+} cells on testes development

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

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

The effect of Dsp knockout in NR5A1^{+} cells on ovarian development

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

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

Dsp knockout in NR5A1$^+$ cells does not affect sex determination

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

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

Discussion

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

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

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

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

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

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

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

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

Declaration of interest

The authors do not have conflict of interest.

Author contributions

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

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References

Albrecht KH & Eicher EM 2001 Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. *Dev Biol* **240** 92-107.

Bornslaeger EA, Corcoran CM, Stappenbeck TS & Green KJ 1996 Breaking the connection: displacement of the desmosomal plaque protein desmoplakin from cell-cell

ACCEPTED MANUSCRIPT - CLEAN COPY

interfaces disrupts anchorage of intermediate filament bundles and alters intercellular junction assembly. *J Cell Biol* **134** 985-1001.

Borum K 1961 Oogenesis in the mouse: a study of the meiotic prophase. *Exp Cell Res* **24** 495-507.

DeFalco T, Takahashi S & Capel B 2011 Two distinct origins for Leydig cell progenitors in the fetal testis. *Dev Biol* **352** 14-26.

Delva E, Tucker DK & Kowalczyk AP 2009 The desmosome. *CSH Perspect Biol* **1** a002543.

Dhillon H, Zigman JM, Ye C, Lee CE, McGovern RA, Tang V, Kenny CD, Christiansen LM, White RD, Edelstein EA, *et al*. 2006 Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* **49** 191-203.

Gallicano GI, Kouklis P, Bauer C, Yin M, Vasioukhin V, Degenstein L & Fuchs E 1998 Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. *J Cell Biol* **143** 2009-2022.

Garrod D & Chidgey M 2008 Desmosome structure, composition and function. *BBA-Biomembranes* **1778** 572-587.

Grive KJ & Freiman RN 2015 The developmental origins of the mammalian ovarian reserve. *Development* **142** 2554-2563.

ACCEPTED MANUSCRIPT - CLEAN COPY

Hu YC, Okumura LM & Page DC 2013 Gata4 is required for formation of the genital ridge in mice. *PLoS Genet* **9** e1003629.

Huang K, Wang Y, Zhang T, He M, Sun G, Wen J, Yan H, Cai H, Yong C & Xia G 2018 JAK signaling regulates germline cyst breakdown and primordial follicle formation in mice. *Biol Open* **7** bio029470.

Ikeda Y, Shen WH, Ingraham HA & Parker KL 1994 Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. *Mol Endocrinol* **8** 654-662.

Kiernan JA 1990 *Histological and Histochemical Methods: Theory and Practice. 2nd ed.* Pergamon Press, Oxford, New York, Seoul, Tokyo.

Kowalczyk AP & Green KJ 2013 Structure, function, and regulation of desmosomes. *Prog Mol Biol Transl Sci* **116** 95-118.

Liu C, Peng J, Matzuk MM & Yao HHC 2015 Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells. *Nat Commun* **6** 6934.

McFarlane L, Truong V, Palmer JS & Wilhelm D 2013 Novel PCR assay for determining the genetic sex of mice. *Sex Dev* **7** 207-211.

McLaren A 2003 Primordial germ cells in the mouse. *Dev Biol* **262** 1-15.

ACCEPTED MANUSCRIPT - CLEAN COPY

Mruk DD & Cheng CY 2011 Desmosomes in the testis: Moving into an unchartered territory. *Spermatogenesis* **1** 47-51.

Norgett EE, Hatsell SJ, Carvajal-Huerta L, Ruiz Cabezas JC, Common J, Purkis PE, Whittock N, Leigh IM, Stevens HP & Kelsell DP 2000 Recessive mutation in desmoplakin disrupts desmoplakin–intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma. *Hum Mol Genet* **9** 2761-2766.

Pang H, Rowan BG, Al-Dhaheri M & Faber LE 2004 Epidermal growth factor suppresses induction by progestin of the adhesion protein desmoplakin in T47D breast cancer cells. *Breast Cancer Res* **6** R239.

Papagerakis S, Shabana AH, Pollock BH, Papagerakis P, Depondt J & Berdal A 2009 Altered desmoplakin expression at transcriptional and protein levels provides prognostic information in human oropharyngeal cancer. *Hum Pathol* **40** 1320-1329.

Peluso JJ, Pappalardo A & Fernandez G 2001 E-cadherin-mediated cell contact prevents apoptosis of spontaneously immortalized granulosa cells by regulating Akt kinase activity. *Biol Reprod* **64** 1183-1190.

Pepling ME & Spradling AC 2001 Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev Biol* **234** 339-351.

ACCEPTED MANUSCRIPT - CLEAN COPY

Pepling ME, Sundman EA, Patterson NL, Gephardt GW, Medico L & Wilson KI 2010 Differences in oocyte development and estradiol sensitivity among mouse strains. *Reproduction* **139** 349-357.

Piprek RP 2009 Molecular mechanisms underlying female sex determination–antagonism between female and male pathway. *Folia Biol-Krakow* **57** 105-113.

Piprek RP, Kloc M & Kubiak JZ 2016 Early development of the gonads: origin and differentiation of the somatic cells of the genital ridges. *Results Probl Cell Differ* **58** 1-22.

Piprek RP, Kloc M, Tassan JP & Kubiak JZ 2017b Development of *Xenopus laevis* bipotential gonads into testis or ovary is driven by sex-specific cell-cell interactions, proliferation rate, cell migration and deposition of extracellular matrix. *Dev Biol* **432** 298-310.

Piprek RP, Kolasa M, Podkowa D, Kloc M & Kubiak JZ 2017a Cell adhesion molecules expression pattern indicates that somatic cells arbitrate gonadal sex of differentiating bipotential fetal mouse gonad. *Mech Dev* **147** 17-27.

Piprek RP, Kolasa M, Podkowa D, Kloc M & Kubiak JZ 2019a N-Cadherin Is Critical for the Survival of Germ Cells, the Formation of Steroidogenic Cells, and the Architecture of Developing Mouse Gonads. *Cells* **8** 1610.

Piprek RP, Kolasa M, Podkowa D, Kloc M & Kubiak JZ 2019b Tissue-specific knockout of E-cadherin (Cdh1) in developing mouse gonads causes germ cells loss. *Reproduction* **158** 147-157.

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Svingen T & Koopman P 2013 Building the mammalian testis: origins, differentiation, and assembly of the component cell populations. *Genes Dev* **27** 2409-2426.

Svingen T, Spiller CM, Kashimada K, Harley VR & Koopman P 2009 Identification of suitable normalizing genes for quantitative real-time RT-PCR analysis of gene expression in fetal mouse gonads. *Sex Dev* **3** 194-204.

Tran NL, Adams DG, Vaillancourt RR & Heimark RL 2002 Signal transduction from N-cadherin increases Bcl-2. Regulation of the phosphatidylinositol 3-kinase/Akt pathway by homophilic adhesion and actin cytoskeletal organization. *J Biol Chem* **277** 32905-32914.

Ungewitter EK & Yao HH 2013 How to make a gonad: cellular mechanisms governing formation of the testes and ovaries. *Sex Dev* **7** 7-20.

Vasioukhin V, Bowers E, Bauer C, Degenstein L & Fuchs E 2001 Desmoplakin is essential in epidermal sheet formation. *Nat Cell Biol* **3** 1076.

Wan H, South AP & Hart IR 2007 Increased keratinocyte proliferation initiated through down regulation of desmoplakin by RNA interference. *Exp Cell Res* **313** 2336-2344.

Western PS, Miles DC, van den Bergen JA, Burton M & Sinclair AH 2008 Dynamic regulation of mitotic arrest in fetal male germ cells. *Stem Cells* **26** 339-347.

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Yang L, Chen Y, Cui T, Knosel T, Zhang Q, Albring KF, Huber O & Petersen I 2012 Desmoplakin acts as a tumor suppressor by inhibition of the Wnt/beta-catenin signaling pathway in human lung cancer. *Carcinogenesis* **33** 1863-1870.

Figure legends

Fig. 1. *Cre* and *Dsp* expression. A. The expression of *Cre* recombinase in XY gonads is high from E11.5 onward. **B**, **C**. Immunostaining of CRE recombinase at E18.5 shows a lack of CRE recombinase in the control testis (*Nr5a1-Cre*$^{-}$*Dsp*$^{+/+}$). The knockout testis (*Nr5a1-Cre*$^{+}$*Dsp*$^{fl/fl}$) express CRE in Sertoli cells (arrowheads) and interstitial cells (arrows). **D**. The expression of *Cre* recombinase in XX gonads is high from E11.5 onward. **E**, **F**. Immunostaining of CRE recombinase at E18.5. The control ovary (*Nr5a1-Cre*$^{-}$*Dsp*$^{+/+}$) does not express Cre. The somatic cells (arrows) of the knockout (*Nr5a1-Cre*$^{+}$*Dsp*$^{fl/fl}$) express Cre. **G**. The expression of *Dsp* is significantly lower (from E11.5 onward) in the knockout XY gonads than in control. **H**, **I**. Immunostaining of desmoplakin in the testes at E18.5. Desmoplakin is present in the control (**H**) but not in the knockout testis (**I**). **J**. The expression of *Dsp* is significantly lower (from E11.5 onward) in the knockout XX gonads than in control. **K**, **L**. Immunostaining of desmoplakin in the ovaries at E18.5. Desmoplakin is present in the control ovary (**K**) but not in the knockout ovary (**L**). *$P<0.05$, n - number of individuals tested, bars indicate standard deviation. Scale bar: 25 μm.

Fig. 2. Gonad size and the number of germ cells. A. The knockout XY and XX gonads are smaller than the control starting from E16.5 onward. **B**. Germ cell number in the knockout XY and XX gonads is lower than the control starting from E16.5 onward. **C**, **D**. Immunostaining of GCNA1 (germ cell marker) shows many germ cells in the control testis

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

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

Fig. 4. Histology of the testes in control and desmoplakin knockout mice. A-D. Histological staining shows a similar gross morphology of control ($Nr5a1\text{-}cre^{+}Dsp^{+/+}$) and knockout testes ($Nr5a1\text{-}cre^{+}Dsp^{fl/fl}$) at stage E18.5. However, in the knockout testis, testis cords (ts) contain only a few germ cells (g) enclosed by Sertoli cells (Sc), while in control, testis cords (ts) have many germ cells (g). In the knockout and control testes, the Sertoli cells

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

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

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

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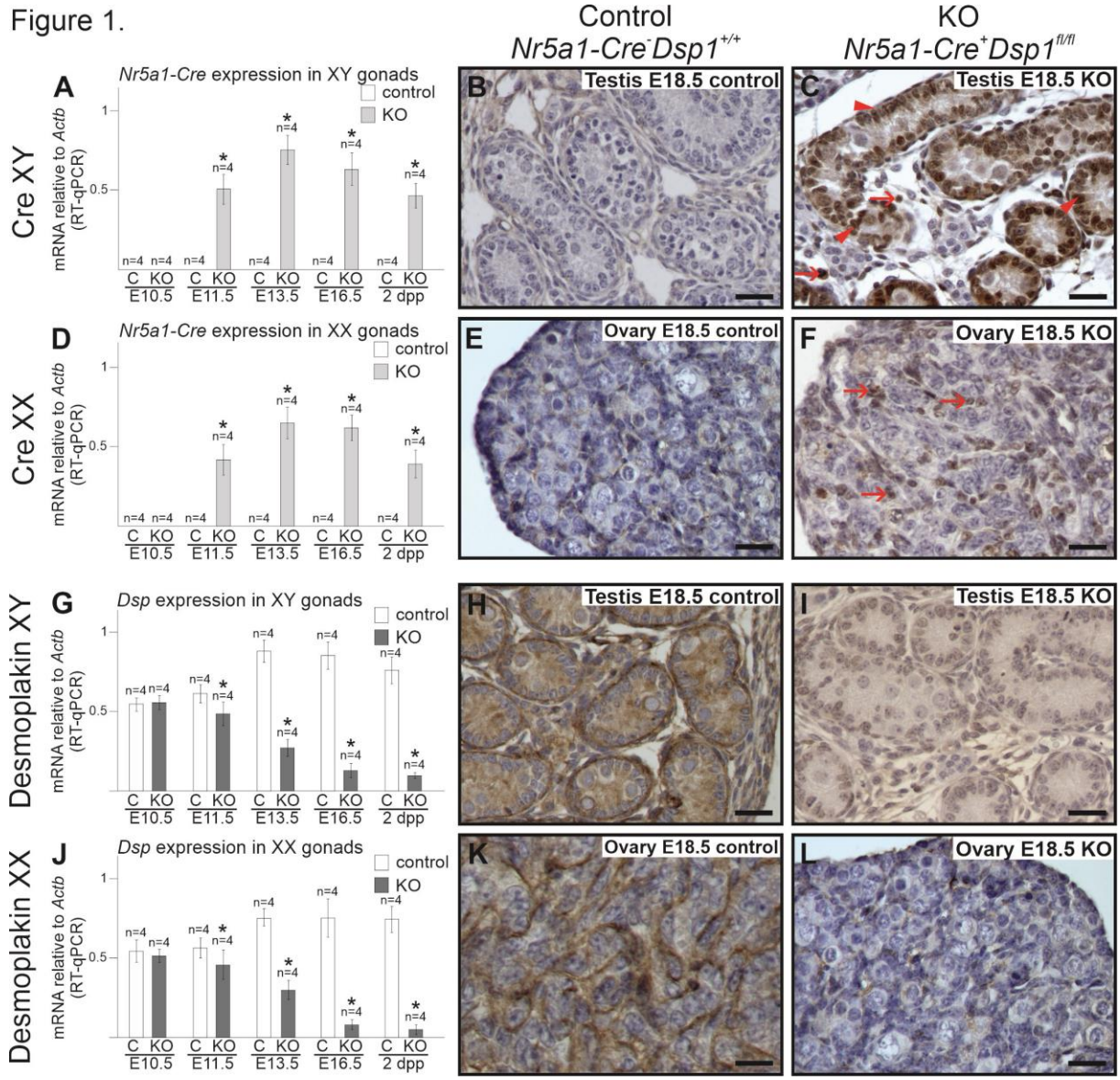
Figure 1.



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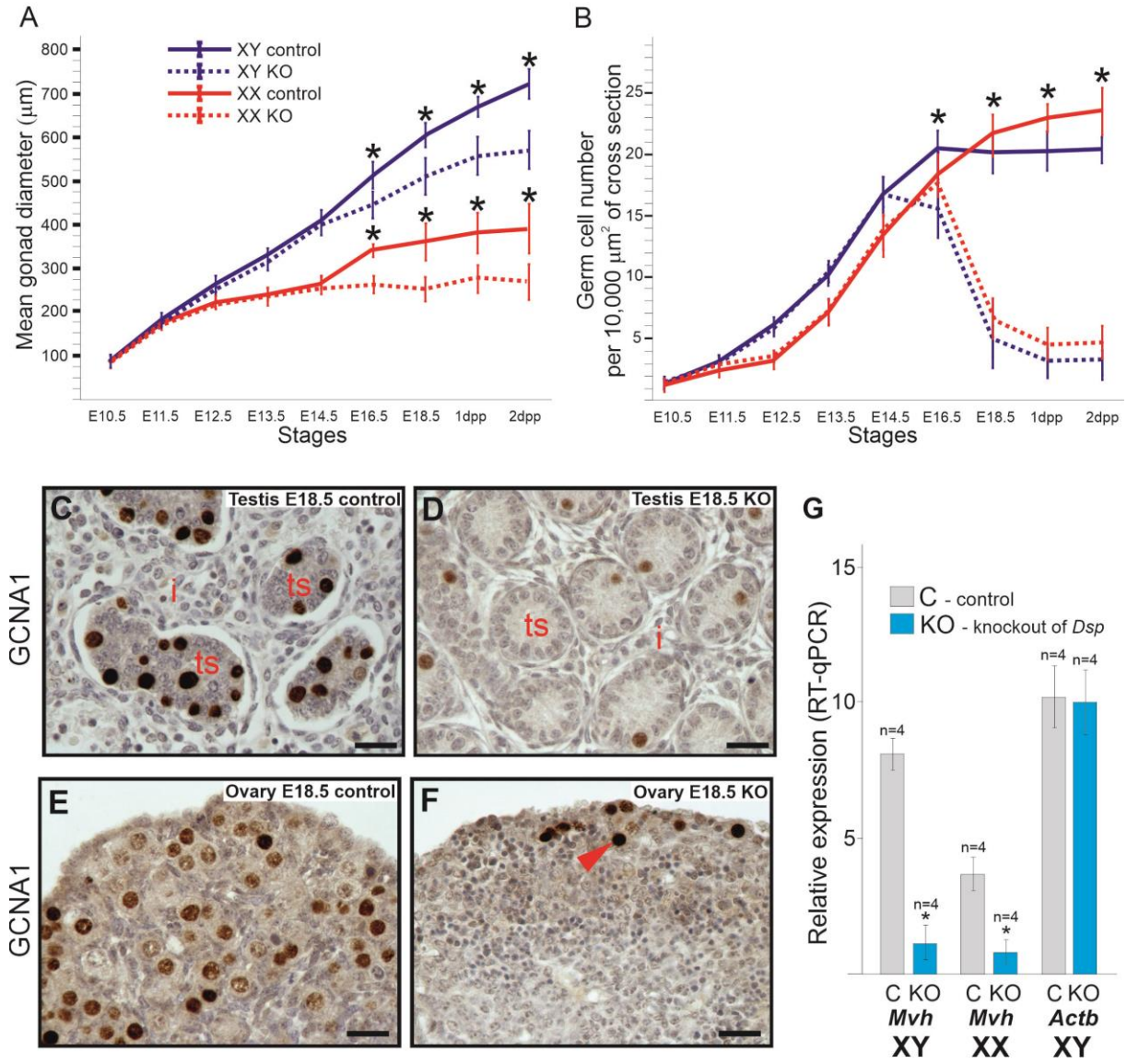

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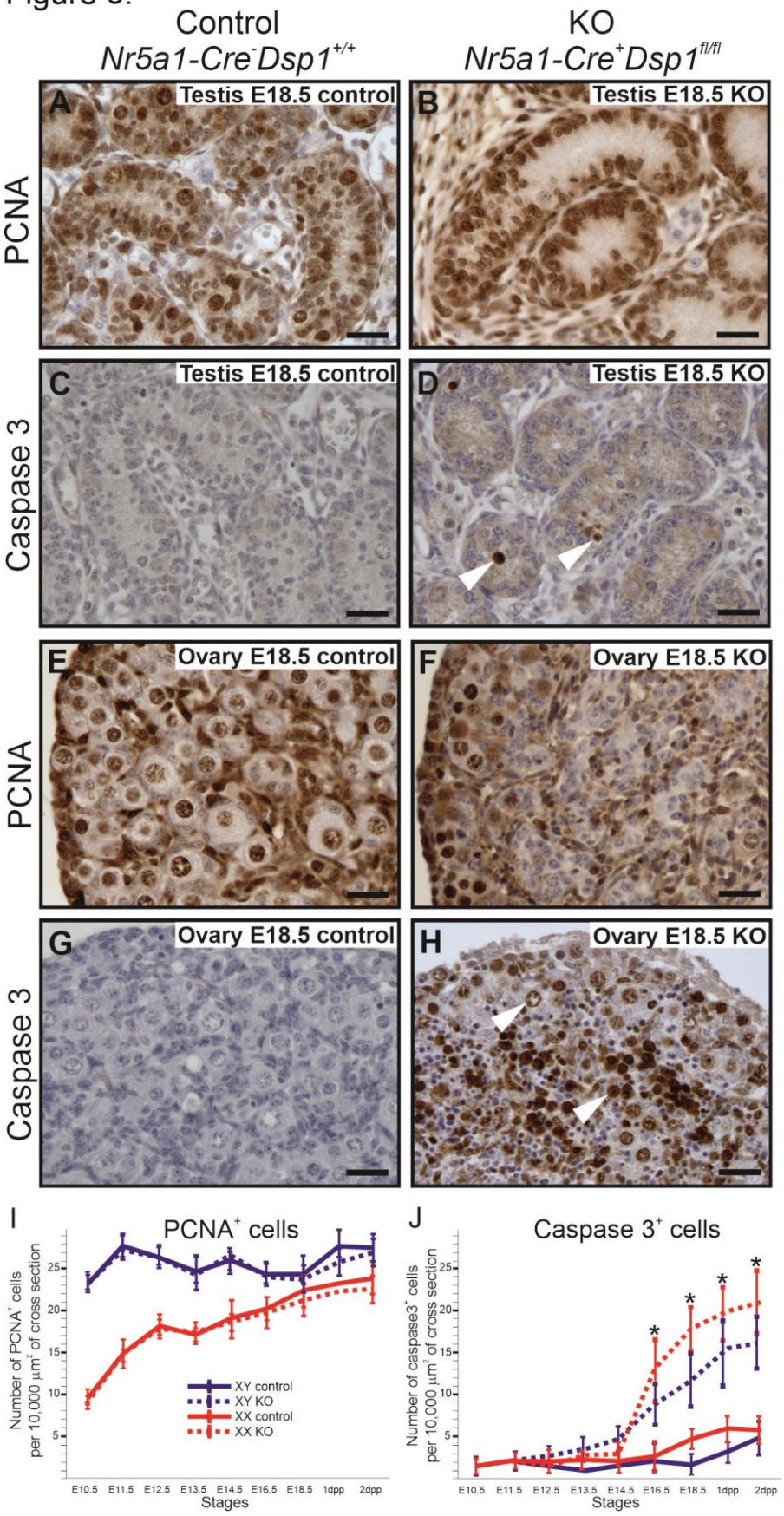

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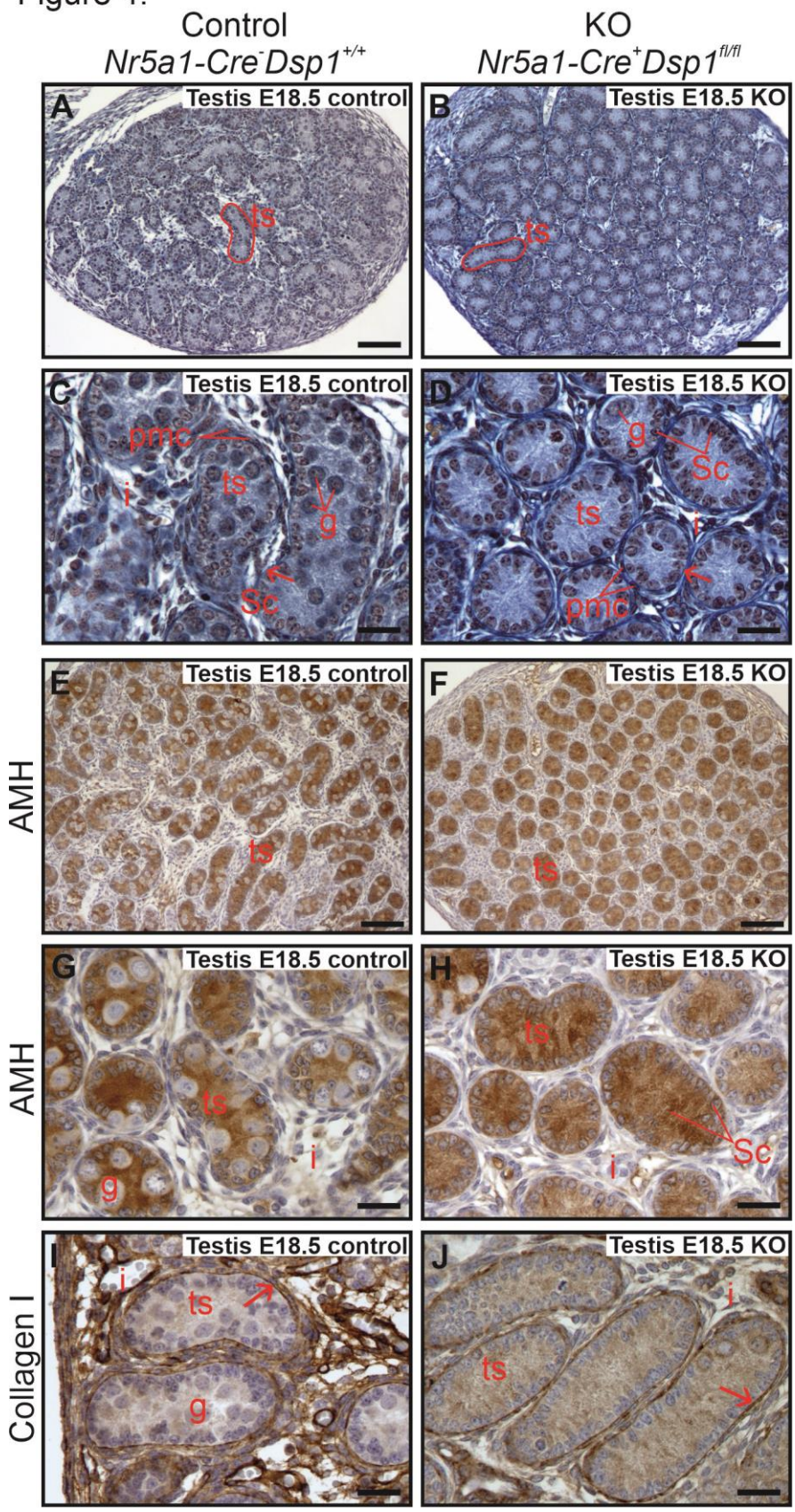

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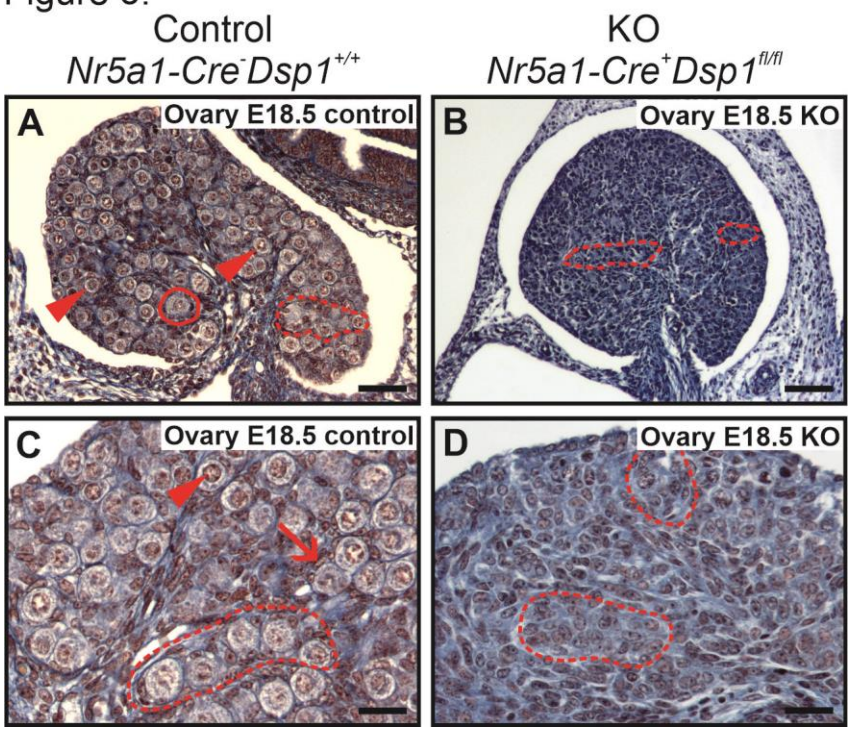

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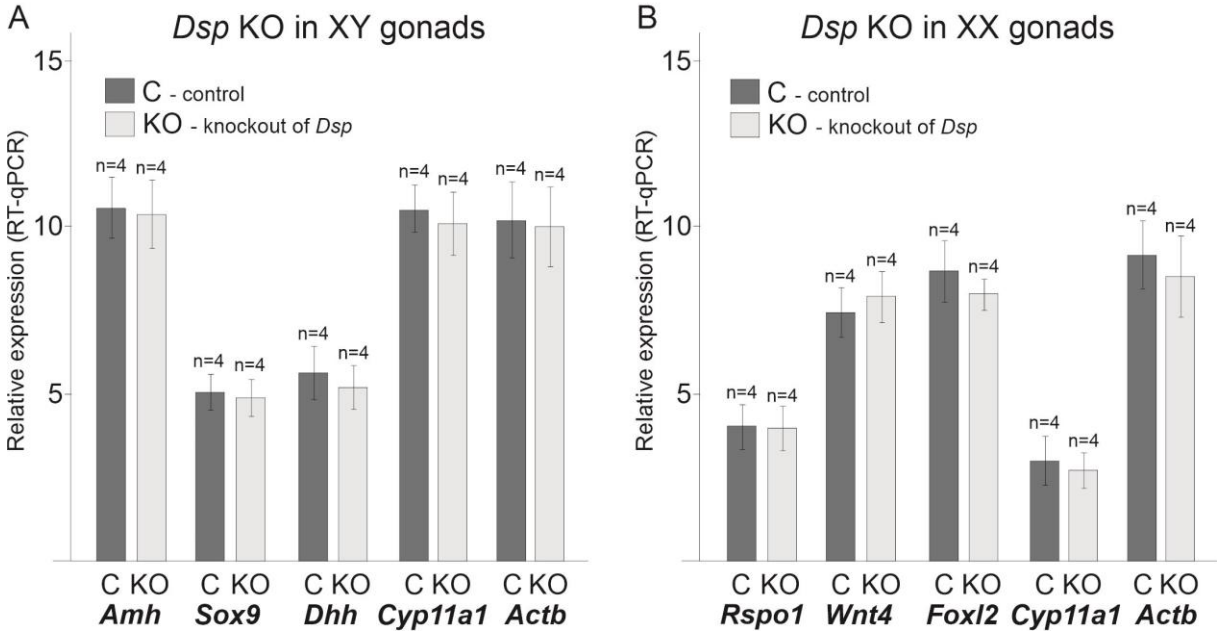

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Table 1. Number of specimens tested.

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	
Nr5a1-Cre $^{-}$ ***Dsp*** $^{+/+}$ **control**	8	8	8	8	8	8	8	8	10	10	8	8	10	10	8	8	8	8	152
Nr5a1-Cre $^{+}$ ***Dsp*** $^{fl/fl}$ **knockout**	8	8	8	8	8	8	8	8	10	10	8	8	10	10	8	8	8	8	152

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

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

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