DMRT1 is a Testis Determining Gene in Rabbits and is Also Essential for Female Fertility

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Abstract:

In mammals, the functions of DMRT1 in sex determination are still ambiguous. To question its role in a non-rodent species, our laboratory generated a line of *DMRT1* mutant rabbits. Throughout gonadal development, XY *DMRT1*$^{-/-}$ fetuses developed ovaries. Interestingly, some SOX9 protein remained detectable in the XY *DMRT1*$^{-/-}$ gonads even though its targets were not activated. This suggests that either these protein levels were insufficient and/or SOX9 could not act in the absence of DMRT1, which is suspected to be its pioneer factor. After birth, both XY and XX *DMRT1*$^{-/-}$ gonads did not undergo meiosis and folliculogenesis. Adult females were infertile, showing that DMRT1 is also crucial for female fertility.

Introduction:

DMRT1 (Doublesex and Mab-3 Related Transcription factor 1) belongs to the highly conserved family of DM domain proteins, which exhibits a zinc finger DNA-binding motif that was initially identified in *Drosophilia* and *Caenorhabditis elegans* (*1*, *2*). Some of its orthologs have been described as Testis Determining Factor (TDF) in vertebrate species such as medaka (*Oryzias latipes)* (*3*), xenope (*Xenopus laevis)* (*4*), or chicken (*5*). In this last, the Z chromosome carries the *DMRT1* gene. In ZZ males, two copies of the *DMRT1* gene are required to induce testis determination. In ZW females and ZZ chickens harboring a non-functional copy, gonads differentiate as ovaries showing that sex determination is based on DMRT1 dosage (*6*).

In mammals, where the sex-determining system is XX/XY, the TDF is the *SRY* gene (Sex determining Region of the Y chromosome) carried by the Y chromosome. Based on the mouse species, DMRT1 does not appear to have retained a crucial function in testis determination since targeted deletion of *Dmrt1* only affects post-natal testis function. Indeed, DMRT1 has roles in both germ cells and supporting cells in the testis, and *Dmrt1*$^{-/-}$ males showed spermatogenesis failure with spermatogonia that did not undergo meiosis (*7*). However, specific knock-out of *Dmrt1* in adult Sertoli cells led to their transdifferentiation into granulosa cells (*8*). Thus, despite losing its function in early testis determination in mice, DMRT1 retained part of this function in adulthood when it is necessary to maintain Sertoli cell identity. Interestingly, an equivalent to DMRT1 was observed in the female. In the ovary, *FOXL2* (Forkhead family box L2) is expressed in female supporting cells very early in development. In mice, *Foxl2* does not appear necessary for fetal ovary differentiation (*9*), while it is required in adult granulosa cells to maintain female-supporting cell identity (*10*). In other mammalian species, such as goats, *FOXL2* played earlier functions in the female and was shown to be crucial for ovarian determination. Indeed, FOXL2 loss-of-function, naturally observed in the PIS (Polled Intersex Syndrome) mutation (*11*) or experimentally induced by genome editing in goats (*12*), led to female-to-male sex reversal with the early development of XX testes. Following *FOXL2* absence of expression in the XX mutant gonads (XX PIS$^{-/-}$ or XX *FOXL2*$^{-/-}$), *DMRT1* was up-regulated within days before increased *SOX9* expression, which then directs the differentiation of Sertoli cells and the formation of testicular cords (*13*). These observations in the goat suggested that DMRT1 could retain function in SOX9 activation and, thus, in testis determination in several mammals. In humans, a few mutations affecting *DMRT1* have been described in patients presenting 46, XY DSD (*14–16*). In particular, a heterozygous *de novo* point mutation in the *DMRT1* gene has been identified in a 46, XY individual with complete gonadal dysgenesis (*17*), suggesting that DMRT1 and SRY may be involved in testicular determination.

To clarify DMRT1 functions in non-rodent mammals, we have chosen the rabbit model, where we generated a DMRT1 mutant line thanks to the CRISPR/Cas9 technology. First, we characterized the DMRT1 expression in control gonads, showing that both XY and XX fetal gonads were expressing *DMRT1* before their sexual differentiation. In XY fetuses, *DMRT1* and *SRY* presented partially overlapping territory, and somatic cells expressing both of them harbored SOX9 expression and differentiated into Sertoli cells. Then, thanks to our CRISPR/Cas9 genetically modified rabbit model, we demonstrated that DMRT1 was required for testis differentiation since XY *DMRT1*$^{-/-}$ rabbits showed early male-to-female sex reversal with differentiating ovaries. However, germ cells failed to undergo meiosis, and follicles did not form in XY and XX *DMRT1*$^{-/-}$ mutant ovaries, leading to female infertility. Finally, we could demonstrate that DMRT1 was a testis-determining factor in mammals and that it was also required for female fertility.

Results

DMRT1 is expressed in genital crests of both sexes and just after SRY in XY developing testes.

DMRT1 expression pattern has already been reported by molecular analysis in the rabbit species from 14 d*pc* to adulthood (*18*). We aimed to investigate further the location of the *DMRT1* expression during gonadal development and first at earlier stages of genital crest formation (12-13 days *post-coïtum*, d*pc*) using *in situ* hybridization (ISH). *SRY* expression was already detected at 12 d*pc* and, as expected, was found only in the XY genital ridges, where it was restricted to the medullary part of the gonad (Fig. 1A). By contrast, *DMRT1* was faintly expressed in the gonads of both sexes, in a few cells of the *medulla* under the coelomic epithelium (Fig. 1A). At this stage, only very few germ cells, expressing *POU5F1*, have completed their migration into the genital ridges (Fig. 1A). Twenty-four hours later, at 13 d*pc*, the genital ridges had tripled in size in both sexes, and the territory of *SRY* expression increased within the XY developing testes (Fig. 1B). The number of somatic cells expressing *DMRT1* was also strongly increased in both sexes, with few of them located in the coelomic epithelium (Fig. 1B). In addition, more *POU5F1*-expressing germ cells were detected (5 to 12 per section instead of 1 or 2 at 12 d*pc*) (Fig. 1A-B).

SOX9 is detected in XY medullar cells co-expressing SRY and DMRT1.

At 14 d*pc*, the SOX9 protein was immunodetected in a few cells located in the medullary part of the XY gonad (Fig. 2). Numerous somatic cells of this region also expressed *SRY* and DMRT1 (Fig. 2), and a few ones co-expressed SOX9 and DMRT1 (fig. S1). In contrast, coelomic epithelial cells only expressed DMRT1 (Fig. 2).

One day later, at 15 d*pc*, Sertoli cells that co-express *SRY*, DMRT1, and SOX9 begin to be organized into embryonic cords (Fig. 2). At this stage, coelomic epithelial cells expressed DMRT1 but were negative for *SRY* and SOX9. Furthermore, we observed an islet of cells expressing *SRY* and DMRT1 located in the mesonephros below the boundary with the gonad (Fig. 2, dotted lines). These cells expressed PAX8 (fig. S2) and could correspond to the recently described supporting-like cell population contributing to the *rete testis* in mice (*19*).

From 16 to 18 d*pc*, the development of the testicular cords proceeded. At these two stages, *SRY*, DMRT1, and SOX9 were expressed only in the Sertoli cells, where *SRY* expression began to decrease from 18 d*pc* (Fig. 2). No more DMRT1 expression could be seen in the coelomic epithelial cells, but the tunica albuginea begins to form (Fig. 2), and consequently, the coelomic epithelium will become the surface epithelium.

Persistent expression of DMRT1 in XX gonadal somatic cells until ovigerous nest formation.

As described above, *DMRT1* expression started at 12 d*pc* in the gonadal somatic compartment of both sexes (Fig. 1A). In the female gonads, *DMRT1* remained expressed in all somatic cells, including those of the coelomic epithelium, until 16 d*pc* (Fig. 3A). Interestingly, as in XY gonads, we observed PAX8-positives cells in XX gonads at 15 d*pc* (fig. S2). These cells could contribute to the formation of the *rete ovarii* as in mice (*19*). At 18 d*pc*, *DMRT1* expression decreased but persisted in some cells located in the coelomic epithelium and just below, where ovigerous nest formation occurred. Interestingly, the female *DMRT1*-antagonist gene *FOXL2* began to be expressed between 16 and 18 dpc when *DMRT1* expression decreased (Fig. 3B). Thereafter, at 20 d*pc*, *DMRT1* expression was limited in some somatic cells enclosed in nascent ovigerous nests where some germinal cells also begun to be positive for DMRT1 (Fig. 3C, fig. S3). At this stage, DMRT1 positive territory seems to overlap that

of RSPO1 but not that of FOXL2 located in the loose conjunctive tissue around the ovigerous nests (Fig. 3C).

The testicular formation is impaired in DMRT1 knock-out XY rabbits

To determine the role of DMRT1 in the rabbit species used as a non-rodent mammalian model, we engineered a *DMRT1* knock-out line using the CRISPR/Cas9 system with two guides RNA located in exon 3. The mutation carried by this line is a 47-bp duplication in sense, leading to a frameshift of the open reading frame and a premature stop codon (fig. S4A). This mutation does not affect *DMRT1* transcription but induces a total absence of protein as attested in post-natal gonads by western blot (fig. S4B-C). Thanks to this line, we first analyzed gonadal formation at 20 d*pc*, when the testis and ovary were distinguishable in control animals. Indeed, at this stage, testes appeared with well-formed seminiferous cords, and ovigerous nest formation was clearly in progress in the ovaries (Fig. 4A). At 20 d*pc*, XY *DMRT1*$^{-/-}$ gonads failed to engage testicular differentiation and appeared quite like control ovaries (Fig. 4A). Moreover, ovarian differentiation did not appear to be affected by the loss of *DMRT1* (Fig. 4A). To better characterize the *DMRT1*$^{-/-}$ gonads in XY and XX fetuses, we established the gonadal transcriptome by RNA-sequencing. Heatmap representation of the 3640 differentially expressed genes in at least one of the four genotypes (adjusted p-value <0.05 and |log2FC|>1; Table S1) was clustered into eight groups (#1 to #8, Fig. 4B, Table S2). Clusters #1 and #7, contained 1331 and 315 genes, respectively, which were preferentially expressed in XY control testes. Expression of these genes was decreased in XY *DMRT1*$^{-/-}$ gonads, harboring levels close to that of the female's ovaries (XX control or *DMRT1*$^{-/-}$). On the other hand, clusters #2, #3, and #5 (537, 582, and 464 genes, respectively) were composed of genes preferentially expressed in XX control ovaries, and their expression was increased in XY *DMRT1*$^{-/-}$ gonads. Thus, deep-sequencing transcriptomics confirmed the ovarian fate of XY *DMRT1*$^{-/-}$ gonads and was illustrated by the heatmap representation of the expression of a selection of the main genes involved in sex determination (Fig. 4C).

Expression levels and patterns of the principal actors of gonadal differentiation were confirmed by RT-qPCR, and the location of positive cells was achieved by immunohistochemistry. As expected, *SOX9*, *AMH*, and *DHH* expression levels were decreased in XY *DMRT1*$^{-/-}$ gonads, remaining like those detected in control or *DMRT1*$^{-/-}$ XX ovaries, while *SRY* expression was enhanced in XY *DMRT1*$^{-/-}$ gonads (Fig. 4D). Interestingly, we noticed a slight increase of SOX9-positive cells in XY *DMRT1*$^{-/-}$ gonads compared to XX control or mutant ovaries (fig. S5). By contrast, *FOXL2* and *CYP19A1* expression were increased in XY *DMRT1*$^{-/-}$ gonads to similar levels as those detected in control or mutant ovaries (Fig. 4E). By immunohistochemistry, we detected cells expressing FOXL2 in XY *DMRT1*$^{-/-}$ gonads (fig. S5). Moreover, *RSPO1* expression was increased in XY *DMRT1*$^{-/-}$ gonads, but it remained lower than in control ovaries or in XX *DMRT1*$^{-/-}$ gonads. In these last, the *RSPO1* expression was also lower than in control ovaries, suggesting a regulatory link between DMRT1 and RSPO1 in the female pathway (Fig. 4E).

Germ cells failed to engage meiosis in DMRT1 mutant gonads

After the sex determination process and the first stages of gonad formation, *DMRT1*$^{-/-}$ gonads engage a female fate and differentiate as ovaries, whatever their sex-chromosome constitution, XX or XY. Whereas the *DMRT1* expression began at 18 d*pc* in the XY germinal lineage of control gonads and 20 d*pc* in XX (fig. S3), its expression was abolished in both somatic and germ cells in *DMRT1*$^{-/-}$ mutant gonads (fig. S5). Although XX or XY *DMRT1*$^{-/-}$ gonads continue to develop as ovaries, most germ cells did not engage in the meiotic process. Indeed, in control ovaries at 3 days *post-partum* (d*pp*), most germ cells were in the zygotene stage, showing nuclei with highly condensed chromatin (*18*) (Fig. 5) and were positives for

Ki67, showing their exit from the G0 phase of the cell cycle (fig. S6). In contrast, in *DMRT1*$^{-/-}$ gonads, few germ cells in the preleptoten stage were observed (Fig. 5), and the majority did not express Ki67 but continued to express the pluripotency marker POU5F1 (fig. S6). Subsequently, the rupture of ovarian nests and, consequently, the follicle formation did not occur in *DMRT1*$^{-/-}$ gonads. At 18 d*pp*, folliculogenesis had already started in control ovaries where the first primordial follicles were visible in the deepest cortical part close to the *medulla* (Fig. 5). By contrast, *DMRT1*$^{-/-}$ gonads seemed to be blocked at a pre-meiotic stage, and folliculogenesis failed to occur (Fig. 5). In adults, *DMRT1*$^{-/-}$ gonads were reduced in size (fig. S7), no germ cells were detected, and some somatic cells evolved toward luteinized cells (Fig. 5). Consequently, both XY and XX females were completely infertile in adulthood.

Discussion

Our study gave new insights into the conservation of the sex-determination genetic cascade across evolution. Although the signal controlling this process could take different forms in metazoans, several downstream transcription factors involved in gonadal differentiation have been conserved through evolution. For instance, *SOX9*, well known in vertebrates as being essential for Sertoli cell differentiation (*20–22*), has a fruit fly ancestor, *Sox100B*, which was found to be necessary for testis development in Drosophila (*23*). However, the most conserved sex-differentiating factor through evolution is DMRT1. Indeed, it has been maintained at the head of the sex determination cascade in reptiles (*24*), fishes (*3*), and birds (*5*). Nevertheless, its functions could have been reduced in mammals since testis differentiates in the absence of DMRT1 (*Dmrt1*$^{-/-}$) in mice (*25*). Our results highlight an evolutionary continuum of this gene in testis determination from birds to rabbits and certainly to non-rodent mammals in general. Interestingly, even *DMRT1* dosage sensibility has been conserved between chicken and rabbits since heterozygous *DMRT1*$^{+/-}$ XY male rabbits present secondary infertility with an arrest of spermatogenesis around two years of age (data not shown).

DMRT1 position in the rabbit sex-determining cascade

As the early stages of gonadal differentiation in rabbits were not fully characterized, we first determined the expressional profiles of the major sex-determining genes in this species, from the early formation of genital crests at 12 d*pc* until 20 d*pc* when the gonadal organization is well fixed and recognizable in both sexes. Interestingly, we observed that DMRT1 expression started at 12 d*pc*, and it was first expressed in the somatic lineage of both sexes, as in mice (*26*, *27*). However, in contrast to mice where *Dmrt1* expression starts at E10.5 in both somatic and germinal compartments, we showed that germline expression was shifted by 6 to 8 days compared to the somatic one in the rabbit male and female gonads, respectively. These differences are undoubtedly related to the timing of gonadal development in rabbits which is longer than in mice and therefore allows better visualization of the different processes. These sequential DMRT1 up-regulations according to cell type and sex also argue in favor of distinct *DMRT1* promoters as already described in rats (*28*). For the somatic XY compartment, *SOX9* expression appears at 14 d*pc* in cells expressing both *DMRT1* and *SRY*, suggesting that both factors are required for *SOX9* up-regulation. This led to the Sertoli cell differentiation and testicular cords formation from 15 d*pc*. In the developing ovary, we showed that *FOXL2* increases when DMRT1 expression starts to shift from somatic cells to germ cells. Moreover, our results suggested DMRT1 involvement in *RSPO1* up-regulation in the ovary.

DMRT1 is required for testis determination in rabbits

In recent years, the advent of new genome editing technologies has made it possible to explore other animal models, such as the goat (*12*) or the rabbit (*29*), and enriching our knowledge on the conservation of ancestral genetic mechanisms in non-rodent mammals. In

rabbits, the CRISPR-Cas9 technology allowed us to generate a null mutation of the *DMRT1* gene, leading to an absence of detectable protein at homozygosity. Thanks to this model, we could demonstrate that DMRT1 kept its leadership in sex determination also in mammals, where SRY stays the "switch-on factor" for testis determination, as previously demonstrated in rabbits (*30*). Very early in fetal life, XY fetuses expressing SRY but lacking DMRT1 (*DMRT1*$^{-/-}$) presented a male-to-female sex reversal. Although SRY expression was maintained in XY homozygous mutant gonads, the activation of *SOX9* expression was weak in the absence of DMRT1. Accordingly, a few cells expressing SOX9 protein were detectable, but SOX9 target genes expression were not activated in XY *DMRT1*$^{-/-}$ gonads. Thus, DMRT1 seems to be required for SRY action on its targets (i.e., *SOX9* gene activation) but also for SOX9 functions in the early fetal gonad. Interestingly, a recent study proposed that DMRT1 can act as a SOX9 pioneer factor in the post-natal testis for Sertoli cell identity maintenance (*31*). In rabbits, DMRT1 is required for SOX9 and SRY functions, and we hypothesize that DMRT1 might be a pioneer factor for both. In the differentiating genital crest, DMRT1 would be required to increase chromatin accessibility on specific sex-related regions, allowing SRY to bind and activate its targets and particularly the expression of *SOX9*. The crucial region for SRY binding was identified in mice more than 500 kb upstream of the *Sox9* transcription start site and named Enhancer 13 (*32*). Conservation studies identified the homolog of Enhancer 13 in many mammalian species, including humans, cows, and rabbits, and DMRT1 consensus sites were predicted in all mammals examined except mice and rats (*32*). In non-rodent mammals, DMRT1 might be required for chromatin remodeling on the Enhancer 13 region to enable SRY binding and *SOX9* expression since the beginning of testis differentiation. In the mouse, which evolved more rapidly, DMRT1 would no longer be necessary for SRY action because the chromatin state of the fetal supporting cells would be more permissive. This could also explain why DMRT1 does not exert any critical function in the fetal testis in mice (*25*). In contrast, it is required for the action of SOX9 in the post-natal testis (*31*), where a sex-specific epigenetic signature was observed (*33*).

DMRT1 is required for germ cell meiosis and female fertility.

In addition to its functions in testis differentiation, DMRT1 also plays a crucial role in the female gonad. Indeed, germ cells did not undergo meiosis in *DMRT1*$^{-/-}$ ovaries, and in the absence of oocyte I, germ cell cysts do not break, compromising follicle formation and female fertility. This specific phenotype is highly similar to those observed in ZW chicken ovaries lacking DMRT1 (*6*) but is quite different from those described in mice. Indeed in mice, even fewer follicles were observed in *Dmrt1*$^{-/-}$ ovaries, female remains fertile (*34*). Interestingly in humans, one case incriminating *DMRT1* in premature ovarian failure has been reported (*35*).

In rabbit fetuses, DMRT1 expression was first detected in differentiating ovarian somatic cells, at least until *FOXL2* up-regulation. However, DMRT1 has also been observed in fetal germ cells from 20 d*pc* until meiosis proceeded after birth. Consequently, germ cell pre-meiotic arrest in *DMRT1*$^{-/-}$ XX gonads could result from *DMRT1* loss-of-function in the germinal or the somatic compartment or both. In the somatic compartment, the absence of DMRT1 in XX homozygous mutants did not seem to disturb the first steps of ovarian differentiation. Nevertheless, deep sequencing transcriptomics revealed dysregulation of the expression of a few genes involved in the WNT/beta-catenin pathway. In particular, *RSPO1,* which is a positive regulator of the WNT signaling, was reduced, and *DKK1,* which is a negative regulator, was increased (Tables S1-S2). These two events could have the effect of limiting the beta-catenin action in both somatic and germinal ovarian cells at the beginning of their differentiation, and this pathway has proven to be crucial in mice to promote germ cell meiosis (*36*). Nevertheless, it cannot be the main event explaining the pre-meiotic failure, and the functions of DMRT1 in germ cells are more certainly incriminated. In mice, DMRT1 was shown to be involved in *Stra8* up-regulation in female germ cells and was thus related to the meiotic process (*34*). This regulatory action also seems to be done in close collaboration with

the retinoic acid pathway (*37*). The sole action of DMRT1 on *STRA8* up-regulation cannot explain the phenotype observed in rabbits where the germ cell seems to be unable to leave their pluripotency stage. It has also been demonstrated in male mice that DMRT1 acts as a regulator of spermatogonia pluripotency by directly regulating different pluripotency-associated genes, including POU5F1 (*38*, *39*). This path is under exploration in our model in order to try to decipher further the critical role of DMRT1 in the germ line of both sexes.

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Data and materials availability: The datasets generated for this study can be found in the sequence read archive at:

Supplementary Materials

Materials and Methods

Figs. S1 to S7

Tables S1 to S5

References

1. S. E.Erdman, K. C.Burtis, The Drosophila doublesex proteins share a novel zinc finger related DNA binding domain. *EMBO J*. **12**, 527–535 (1993).
2. C. S. Raymond, C. E. Shamu, M. M. Shen, K. J. Seifert, B. Hirsch, J. Hodgkin, D. Zarkower, Evidence for evolutionary conservation of sex-determining genes. *Nature*. **391**, 691–695 (1998).

3. M. Matsuda, Y. Nagahama, A. Shinomiya, T. Sato, C. Matsuda, T. Kobayashi, C. E. Morrey, N. Shibata, S. Asakawa, N. Shimizu, H. Hori, S. Hamaguchi, M. Sakaizumi, DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature*. **417**, 559–563 (2002).
4. S. Yoshimoto, N. Ikeda, Y. Izutsu, T. Shiba, N. Takamatsu, M. Ito, Opposite roles of DMRT1 and its W-linked paralogue, DM-W, in sexual dimorphism of Xenopus laevis: implications of a ZZ/ZW-type sex-determining system. *Development*. **137**, 2519–2526 (2010).
5. C. A. Smith, K. N. Roeszler, T. Ohnesorg, D. M. Cummins, P. G. Farlie, T. J. Doran, A. H. Sinclair, The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature*. **461**, 267–271 (2009).
6. J. Ioannidis, G. Taylor, D. Zhao, L. Liu, A. Idoko-Akoh, D. Gong, R. Lovell-Badge, S. Guioli, M. J. McGrew, M. Clinton, Primary sex determination in birds depends on DMRT1 dosage, but gonadal sex does not determine adult secondary sex characteristics. *Proc. Natl. Acad. Sci.* **118** (2021), doi:10.1073/pnas.2020909118.
7. C. K. Matson, M. W. Murphy, M. D. Griswold, S. Yoshida, V. J. Bardwell, D. Zarkower, The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. *Dev. Cell*. **19**, 612–624 (2010).
8. C. K. Matson, M. W. Murphy, A. L. Sarver, M. D. Griswold, V. J. Bardwell, D. Zarkower, DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature*. **476**, 101–104 (2011).
9. M. Uda, C. Ottolenghi, L. Crisponi, J. E. Garcia, M. Deiana, W. Kimber, A. Forabosco, A. Cao, D. Schlessinger, G. Pilia, Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Hum. Mol. Genet.* **13**, 1171–1181 (2004).
10. C. Ottolenghi, S. Omari, J. E. Garcia-Ortiz, M. Uda, L. Crisponi, A. Forabosco, G. Pilia, D. Schlessinger, Foxl2 is required for commitment to ovary differentiation. *Hum. Mol. Genet.* **14**, 2053–2062 (2005).
11. E. Pailhoux, B. Vigier, S. Chaffaux, N. Servel, S. Taourit, J.-P. Furet, M. Fellous, F. Grosclaude, E. P. Cribiu, C. Cotinot, D. Vaiman, A 11.7-kb deletion triggers intersexuality and polledness in goats. *Nat. Genet.* **29**, 453–458 (2001).
12. L. Boulanger, M. Pannetier, L. Gall, A. Allais-Bonnet, M. Elzaiat, D. Le Bourhis, N. Daniel, C. Richard, C. Cotinot, N. B. Ghyselinck, E. Pailhoux, FOXL2 is a female sex-determining gene in the goat. *Curr. Biol. CB*. **24**, 404–408 (2014).
13. M. Elzaiat, L. Jouneau, D. Thépot, C. Klopp, A. Allais-Bonnet, C. Cabau, M. André, S. Chaffaux, E.-P. Cribiu, E. Pailhoux, M. Pannetier, High-throughput sequencing analyses of XX genital ridges lacking FOXL2 reveal DMRT1 up-regulation before SOX9 expression during the sex-reversal process in goats. *Biol. Reprod*. **91**, 153 (2014).
14. M. P. de Mello, F. B. Coeli, J. G. Assumpção, T. M. Castro, A. T. Maciel-Guerra, A. P. Marques-de-Faria, M. T. M. Baptista, G. Guerra-Júnior, Novel DMRT1 3'UTR+11insT mutation associated to XY partial gonadal dysgenesis. *Arq. Bras. Endocrinol. Metabol.* **54**, 749–753 (2010).
15. S. Ledig, O. Hiort, L. Wünsch, P. Wieacker, Partial deletion of DMRT1 causes 46,XY ovotesticular disorder of sexual development. *Eur. J. Endocrinol*. **167**, 119–124 (2012).
16. V. Chauhan, V. P. Jyotsna, V. Jain, R. Khadgawat, R. Dada, Novel Heterozygous Genetic Variants in Patients with 46,XY Gonadal Dysgenesis. *Horm. Metab. Res.* **49**, 36–42 (2017).
17. M. W. Murphy, J. K. Lee, S. Rojo, M. D. Gearhart, K. Kurahashi, S. Banerjee, G.-A. Loeuille, A. Bashamboo, K. McElreavey, D. Zarkower, H. Aihara, V. J. Bardwell, An ancient protein-DNA interaction underlying metazoan sex determination. *Nat. Struct. Mol. Biol.* **22**, 442–451 (2015).
18. N. Daniel-Carlier, E. Harscoët, D. Thépot, A. Auguste, E. Pailhoux, G. Jolivet, Gonad differentiation in the rabbit: evidence of species-specific features. *PloS One*. **8**, e60451 (2013).
19. C. Mayère, V. Regard, A. Perea-Gomez, C. Bunce, Y. Neirijnck, C. Djari, N. Bellido-Carreras, P. Sararols, R. Reeves, S. Greenaway, M. Simon, P. Siggers, D. Condrea, F. Kühne, I. Gantar, F. Tang, I. Stévant, L. Batti, N. B. Ghyselinck, D. Wilhelm, A. Greenfield, B. Capel, M.-C. Chaboissier, S. Nef, Origin, specification and differentiation of a rare supporting-like lineage in the developing mouse gonad. *Sci. Adv*. **8**, eabm0972 (2022).
20. V. P. I. Vidal, M.-C. Chaboissier, D. G. de Rooij, A. Schedl, Sox9 induces testis development in XX transgenic mice. *Nat. Genet.* **28**, 216–217 (2001).
21. M.-C. Chaboissier, A. Kobayashi, V. I. P. Vidal, S. Lützkendorf, H. J. G. van de Kant, M. Wegner, D. G. de Rooij, R. R. Behringer, A. Schedl, Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development*. **131**, 1891–1901 (2004).
22. Y. Qin, C. E. Bishop, Sox9 is sufficient for functional testis development producing fertile male mice in the absence of Sry. *Hum. Mol. Genet.* **14**, 1221–1229 (2005).
23. S. Nanda, T. J. DeFalco, S. H. Y. Loh, N. Phochanukul, N. Camara, M. V. Doren, S. Russell, Sox100B, a Drosophila Group E Sox-domain Gene, Is Required for Somatic Testis Differentiation. *Sex. Dev.* **3**, 26–37 (2009).
24. W. Sun, H. Cai, G. Zhang, H. Zhang, H. Bao, L. Wang, J. Ye, G. Qian, C. Ge, Dmrt1 is required for primary male sexual differentiation in Chinese soft-shelled turtle Pelodiscus sinensis. *Sci. Rep*. **7**, 4433 (2017).

25. C. S. Raymond, M. W. Murphy, M. G. O'Sullivan, V. J. Bardwell, D. Zarkower, Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev*. **14**, 2587–2595 (2000).
26. C. S. Raymond, J. R. Kettlewell, B. Hirsch, V. J. Bardwell, D. Zarkower, Expression of Dmrt1 in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. *Dev. Biol*. **215**, 208–220 (1999).
27. N. Lei, K. I. Hornbaker, D. A. Rice, T. Karpova, V. A. Agbor, L. L. Heckert, Sex-Specific Differences in Mouse DMRT1 Expression Are Both Cell Type- and Stage-Dependent During Gonad Development1. *Biol. Reprod*. **77**, 466–475 (2007).
28. N. Lei, T. Karpova, K. I. Hornbaker, D. A. Rice, L. L. Heckert, Distinct Transcriptional Mechanisms Direct Expression of the Rat Dmrt1 Promoter in Sertoli Cells and Germ Cells of Transgenic Mice1. *Biol. Reprod*. **81**, 118–125 (2009).
29. G. Jolivet, N. Daniel-Carlier, E. Harscoët, E. Airaud, A. Dewaele, C. Pierson, F. Giton, L. Boulanger, N. Daniel, B. Mandon-Pépin, M. Pannetier, E. Pailhoux, Fetal Estrogens are not Involved in Sex Determination But Critical for Early Ovarian Differentiation in Rabbits. *Endocrinology*. **163**, bqab210 (2022).
30. Y. Song, T. Liu, Y. Wang, J. Deng, M. Chen, L. Yuan, Y. Lu, Y. Xu, H. Yao, Z. Li, L. Lai, Mutation of the Sp1 binding site in the 5' flanking region of SRY causes sex reversal in rabbits. *Oncotarget*. **8**, 38176–38183 (2017).
31. R. E. Lindeman, M. W. Murphy, K. S. Agrimson, R. L. Gewiss, V. J. Bardwell, M. D. Gearhart, D. Zarkower, The conserved sex regulator DMRT1 recruits SOX9 in sexual cell fate reprogramming. *Nucleic Acids Res*. **49**, 6144-6164 (2021).
32. N. Gonen, C. R. Futtner, S. Wood, S. A. Garcia-Moreno, I. M. Salamone, S. C. Samson, R. Sekido, F. Poulat, D. M. Maatouk, R. Lovell-Badge, Sex reversal following deletion of a single far upstream enhancer of Sox9. *Science*. **360**, 1469–1473 (2018).
33. S. A. Garcia-Moreno, C. R. Futtner, I. M. Salamone, N. Gonen, R. Lovell-Badge, D. M. Maatouk, Gonadal supporting cells acquire sex-specific chromatin landscapes during mammalian sex determination. *Dev. Biol*. **446**, 168–179 (2019).
34. A. D. Krentz, M. W. Murphy, A. L. Sarver, M. D. Griswold, V. J. Bardwell, D. Zarkower, DMRT1 promotes oogenesis by transcriptional activation of Stra8 in the mammalian fetal ovary. *Dev. Biol*. **356**, 63–70 (2011).
35. I. Bartels, I. Pütz, N. Reintjes, C. Netzer, M. Shoukier, Normal intelligence and premature ovarian failure in an adult female with a 7.6 Mb de novo terminal deletion of chromosome 9p. *Eur. J. Med. Genet*. **56**, 458–462 (2013).
36. M. Le Rolle, F. Massa, P. Siggers, L. Turchi, A. Loubat, B.-K. Koo, H. Clevers, A. Greenfield, A. Schedl, M.-C. Chaboissier, A.-A. Chassot, Arrest of WNT/β-catenin signaling enables the transition from pluripotent to differentiated germ cells in mouse ovaries. *Proc. Natl. Acad. Sci. U. S. A*. **118**, e2023376118 (2021).
37. C.-W. Feng, G. Burnet, C. M. Spiller, F. K. M. Cheung, K. Chawengsaksophak, P. Koopman, J. Bowles, Identification of regulatory elements required for Stra8 expression in fetal ovarian germ cells of the mouse. *Development*. **148**, dev194977 (2021).
38. A. D. Krentz, M. W. Murphy, S. Kim, M. S. Cook, B. Capel, R. Zhu, A. Matin, A. L. Sarver, K. L. Parker, M. D. Griswold, L. H. J. Looijenga, V. J. Bardwell, D. Zarkower, The DM domain protein DMRT1 is a dose-sensitive regulator of fetal germ cell proliferation and pluripotency. *Proc. Natl. Acad. Sci. U. S. A*. **106**, 22323–22328 (2009).
39. T. Zhang, J. Oatley, V. J. Bardwell, D. Zarkower, DMRT1 Is Required for Mouse Spermatogonial Stem Cell Maintenance and Replenishment. *PLoS Genet*. **12**, e1006293 (2016).
40. M. Peyny, P. Jarrier-Gaillard, L. Boulanger, N. Daniel, S. Lavillatte, V. Cadoret, P. Papillier, D. Monniaux, N. Peynot, V. Duranthon, G. Jolivet, R. Dalbies-Tran, Investigating the role of BCAR4 in ovarian physiology and female fertility by genome editing in rabbit. *Sci. Rep*. **10**, 4992 (2020).
41. G. Jolivet, S. Braud, B. DaSilva, B. Passet, E. Harscoët, C. Viglietta, T. Gautier, L. Lagrost, N. Daniel-Carlier, L.-M. Houdebine, I. Harosh, Induction of Body Weight Loss through RNAi-Knockdown of APOBEC1 Gene Expression in Transgenic Rabbits. *PLOS ONE*. **9**, e106655 (2014).
42. A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T. R. Gingeras, STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. **29**, 15–21 (2013).
43. Y. Liao, G. K. Smyth, W. Shi, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. **30**, 923–930 (2014).
44. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. **15**, 550 (2014).
45. Y. Benjamini, Y. Hochberg, Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B Methodol*. **57**, 289–300 (1995).
46. D. B. Craig, S. Kannan, A. A. Dombkowski, Augmented annotation and orthologue analysis for Oryctolagus cuniculus: Better Bunny. *BMC Bioinformatics*. **13**, 84 (2012).

Figure Legends

Fig. 1. *SRY, DMRT1,* and *POU5F1* location during early gonadal development. Location of *SRY*, *DMRT1,* and *POU5F1* by *in situ* hybridization (RNAscope technology) on XY and XX control gonads at (**A**) 12 days *post-coïtum* (d*pc*) or (**B**) 13 d*pc*. Dotted line: developing genital crests. Arrowheads: coelomic epithelial cells expressing *DMRT1*. Scale bar = 50 µm.

Fig. 2. Somatic markers location during testis differentiation. Location of *SRY* by *in situ* hybridization (RNAscope technology), DMRT1, and SOX9 by immunohistochemistry on XY control testes from 14 to 18 d*pc*. Dotted line at 15 d*pc*: territory with cells expressing *SRY* and DMRT1 but not SOX9. Arrowheads: tunica albuginea in formation. Scale bar = 50 µm.

Fig. 3. Somatic markers location and expression during ovarian differentiation. (**A**) Immunostaining of DMRT1 on XX control gonads from 14 to 18 d*pc*. (**B**) RT-qPCR analyses of *RSPO1*, *DMRT1*, and *FOXL2* expression from 16 to 20 d*pc* in control gonads of both sexes (n=3-5). (C) *RSPO1 in situ* hybridization (RNAscope technology), immunostaining of DMRT1 and FOXL2 on 20 d*pc* control ovary. Scale bar = 50 µm.

Fig. 4. Ovarian-like morphology and transcriptomic signature of XY *DMRT1*$^{-/-}$ gonads at 20 d*pc*. (**A**) Hematoxylin and eosin staining of gonads sections from control and *DMRT1*$^{-/-}$ 20 d*pc* rabbits. The enlarged area shows the characteristic ovarian surface epithelium found on XY *DMRT1*$^{-/-}$ gonads. Scale bar = 50 µm. Heatmap representation of (**B**) 3460 deregulated genes (adjusted p-value <0.05 and |log2FC|>1) or (**C**) 27 selected genes between XY control, XY *DMRT1*$^{-/-}$, XX *DMRT1*$^{-/-}$, and XX control at 20 d*pc*. RT-qPCR analyses of (**D**) testicular-related differentiation genes (*SOX9*, *AMH*, *DHH*, and *SRY*) or (**E**) ovarian-related differentiation genes (*FOXL2*, *CYP19A1*, and *RSPO1*) in XY control, XY *DMRT1*$^{-/-}$, XX *DMRT1*$^{-/-}$ and XX control gonads (n=4-5) at 20 d*pc*. Statistical analyses were performed using the non-parametric Kruskal-Wallis test, followed by a pairwise permutation test. Significant differences are indicated by different letters.

Fig. 5. Evolution of gonadal morphogenesis in XY and XX *DMRT1*$^{-/-}$ rabbits. Hematoxylin and eosin staining of gonad sections from XY and XX *DMRT1*$^{-/-}$ gonads and XX control ovaries at 3 days *post-partum* (d*pp*), 18 d*pp*, and in adulthood (4-9 months). The enlargements for the first two panels correspond to the nuclei pointed by an arrow. PL: preleptotene stage; L: leptotene stage; Z: zygotene stage; D: diplotene stage; F: ovarian follicle; CL: luteal cells. Scale bar = 50 µm.

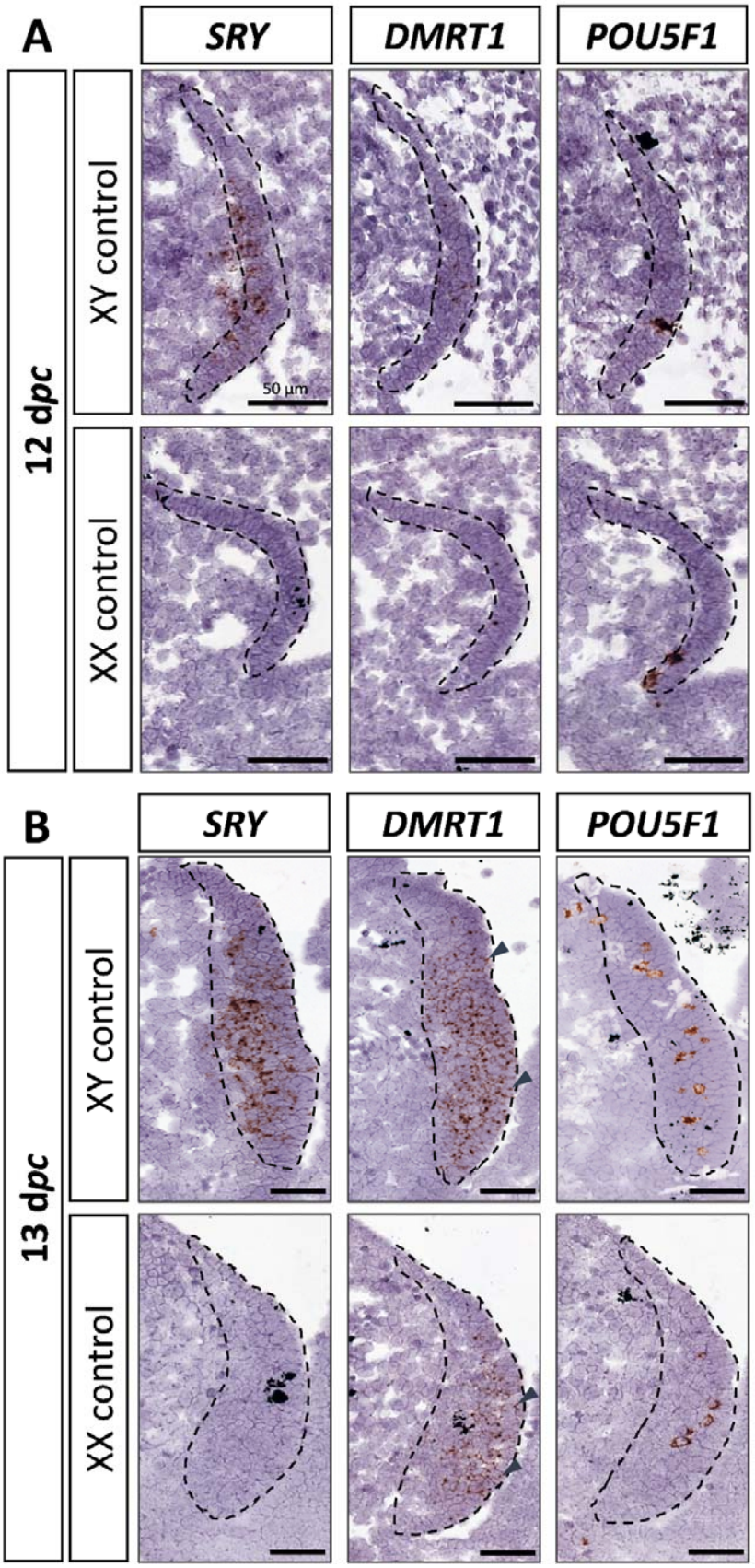

Fig. 1. *SRY*, *DMRT1*, and *POU5F1* location during early gonadal development.

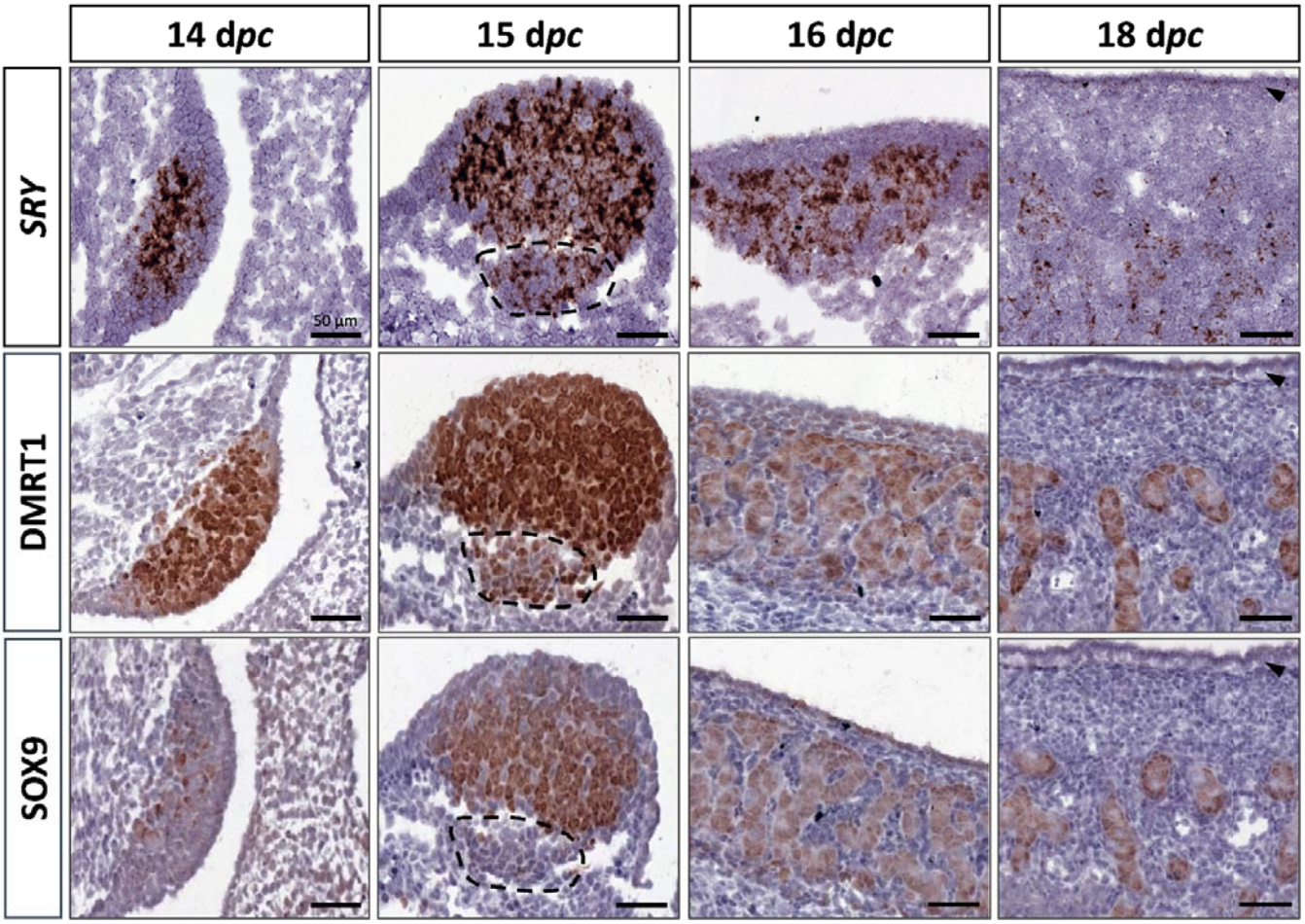

Fig. 2. Somatic markers location during testis differentiation.

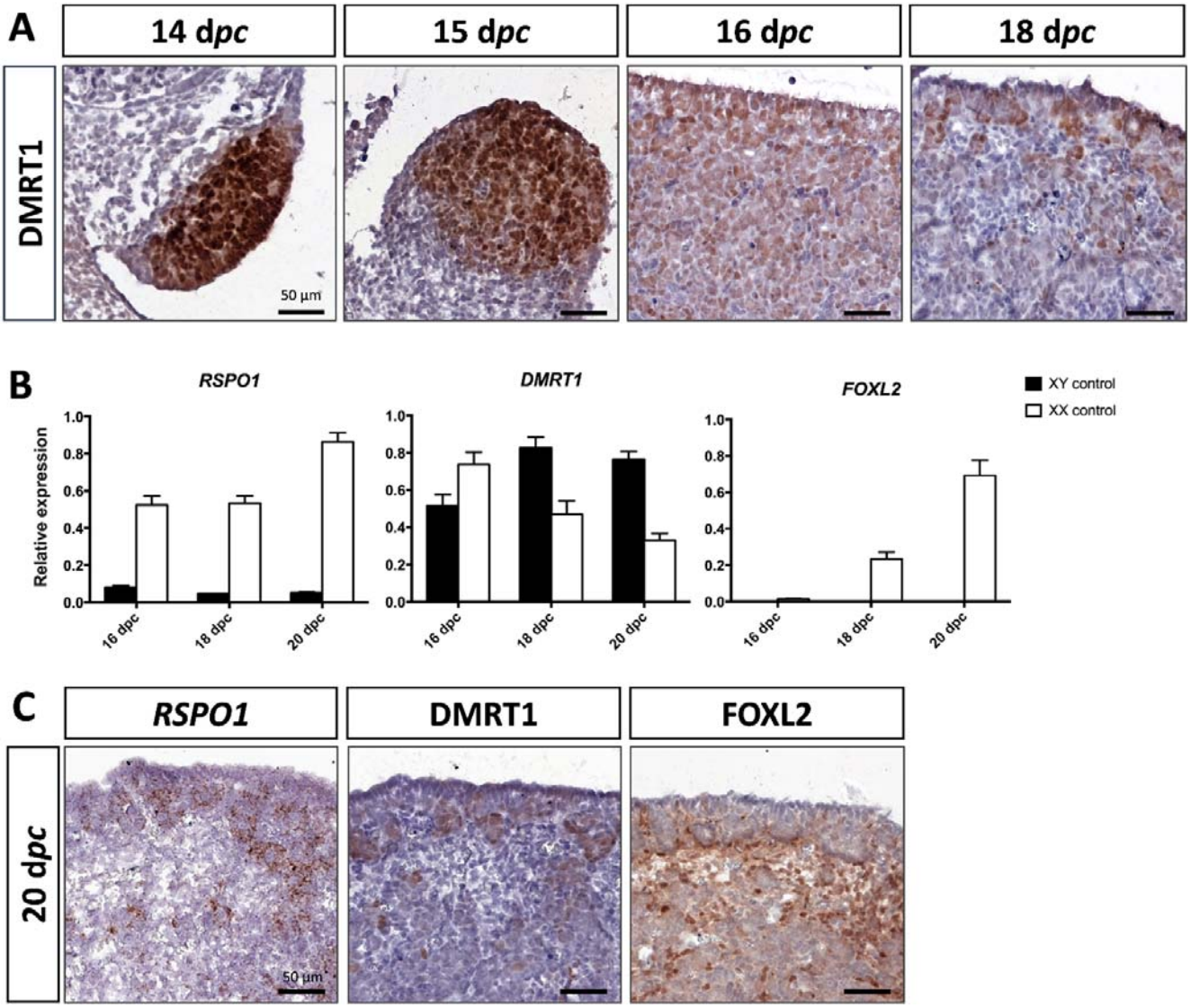

Fig. 3. Somatic markers location and expression during ovarian differentiation.

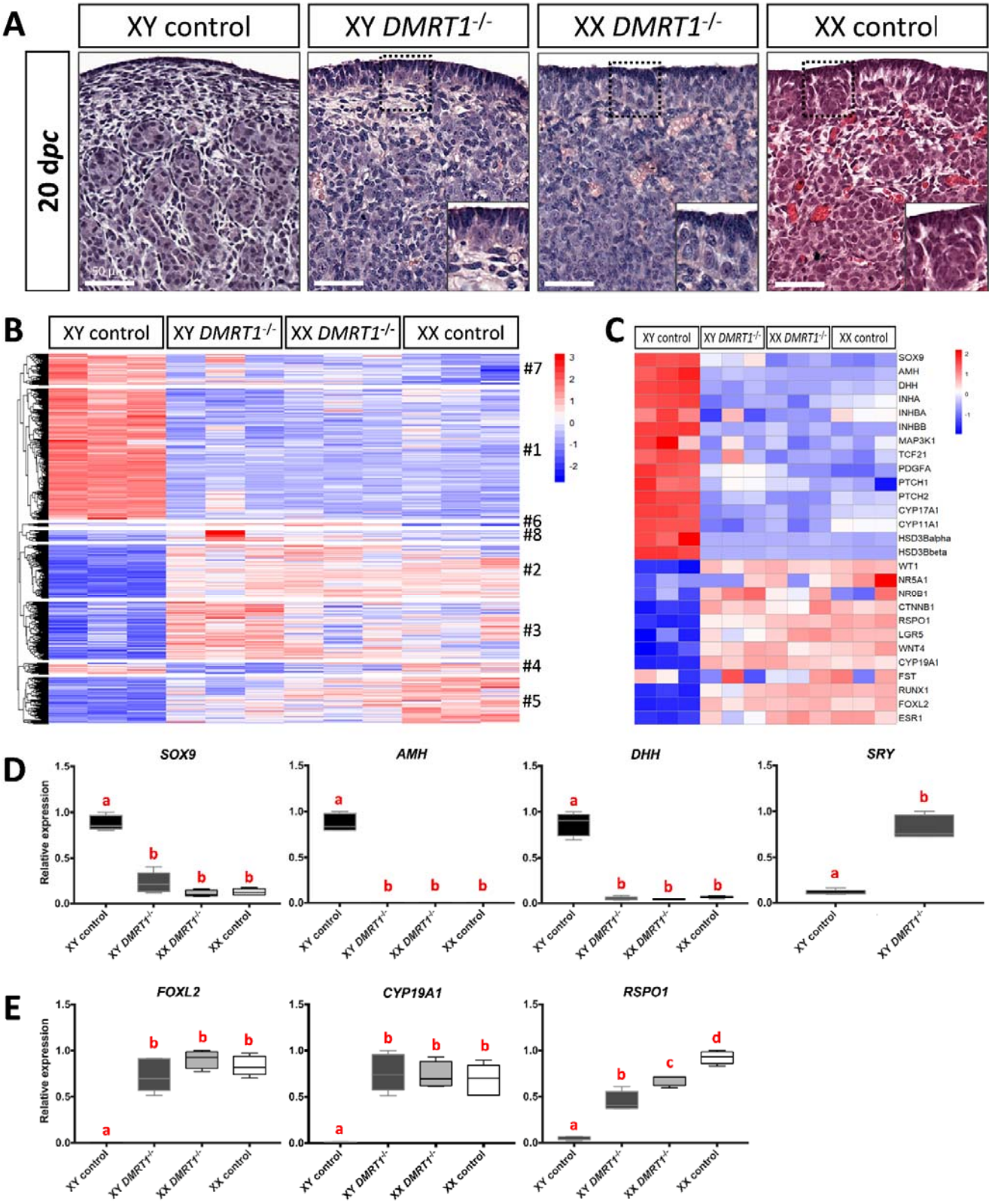

Fig. 4. Ovarian-like morphology and transcriptomic signature of XY *DMRT1*$^{-/-}$ gonads at 20 d*pc*.

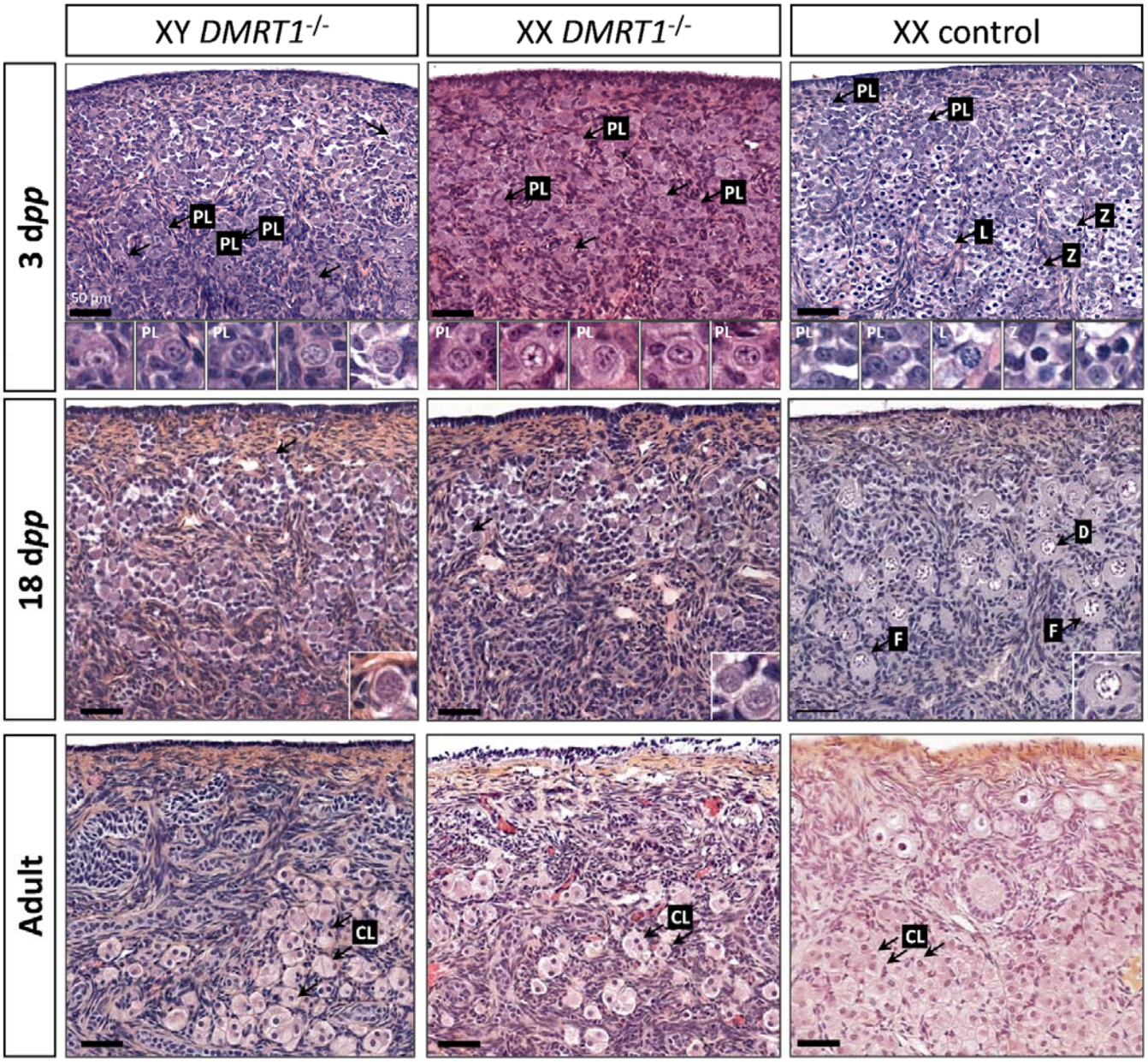

Fig. 5. Evolution of gonadal morphogenesis in XY and XX *DMRT1*$^{-/-}$ rabbits.