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TRIM28-dependent SUMOylation protects the adult ovary from activation of the testicular pathway

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Gonadal sexual fate in mammals is determined during embryonic development and must be actively maintained in adulthood. In the mouse ovary, oestrogen receptors and FOXL2 protect ovarian granulosa cells from transdifferentiation into Sertoli cells, their testicular counterpart. However, the mechanism underlying their protective effect is unknown. Here, we show that TRIM28 is required to prevent female-to-male sex reversal of the mouse ovary after birth. We found that upon loss of *Trim28*, ovarian granulosa cells transdifferentiate to Sertoli cells through an intermediate cell type, different from gonadal embryonic progenitors. TRIM28 is recruited on chromatin in the proximity of FOXL2 to maintain the ovarian pathway and to repress testicular-specific genes. The role of TRIM28 in ovarian maintenance depends on its E3-SUMO ligase activity that regulates the sex-specific SUMOylation profile of ovarian-specific genes. Our study identifies TRIM28 as a key factor in protecting the adult ovary from the testicular pathway.

For long time, it was thought that in mammals, adult gonadal sex assignment was determined and fixed during embryonic development. Any perturbation during this period leads to various disorders of sexual development. However, some teleost fish species display sequential hermaphroditism: gonadal sex is not definitively established in adulthood, and social stimuli can re-assign gonads to the opposite sex (for review see[1]). Moreover, postnatal sex reversal has been observed in several mouse models: ovarian masculinisation upon deletion of oestrogen receptor 1 and 2 (*Esr1-2*)[2] or of *Cyp19a1*[3], as well as after postnatal conditional knock-out (cKO) of *FoxL2*[4] and ectopic ovarian expression of *Dmrt1*[5]. In these cases, the initial cellular event is ovarian-to-testicular transdifferentiation of the supporting cell lineage (granulosa cells to Sertoli cells). Conversely, deletion of *Dmrt1* in postnatal testes[6] or of both *Sox8* and *Sox9*[7] induces Sertoli-to-granulosa cell transdifferentiation. These results indicate that granulosa and Sertoli cells retain the ability to transdifferentiate into the

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opposite sexual fate, and that constant repression of the alternative fate in adult life is required to maintain their cell fate identity and function. However, there is only limited information on the epigenetic and transcriptional programmes implicated in cell fate reprogramming of the supporting lineage.

We previously showed that the epigenetic regulator TRIM28 is a partner of SOX9 in mouse fetal Sertoli cells[8]. TRIM28 is a versatile nuclear scaffold protein that coordinates the assembly of protein complexes containing different chromatin remodelling factors. It can be recruited on chromatin upon interaction with DNA-binding proteins, such as KRAB-ZNF family members[9–11], or with transcription factors[12–14]. TRIM28 was originally associated with transcriptional repression[9] and heterochromatin formation[15,16]; however, many evidences show that it also positively regulates gene expression[12,13,14,17] and controls transcriptional pausing[18,19]. Despite its interaction with SOX9, cKO of *Trim28* in Sertoli cells results in adult males with hypoplastic testes and spermatogenesis defects, but no sex reversal[20]. This suggests that in Sertoli cells, TRIM28 is required to control spermatogenesis, but not for the maintenance of the somatic cell component of the testis.

In this work, to understand its role in ovarian physiology, we generated a cKO of *Trim28* in the somatic compartment of the developing mouse ovary. We observed sex reversal in adult ovaries where the follicular structure progressively reorganised in pseudo-tubules with Sertoli-like cells. We then combined mouse genetic with transcriptomic and genomic approaches to determine the molecular action of TRIM28 and its interplay with FOXL2 in adult ovaries. Our data show that TRIM28 maintains the adult ovarian phenotypes through its SUMO-E3-ligase activity that controls the granulosa cells programme and represses the Sertoli cell pathway.

Results

Deletion of *Trim28* induces masculinisation of adult ovary

Double immunostaining of XX gonads at 13.5 days post-coitum (dpc) showed that TRIM28 is co-expressed with FOXL2 in ovarian pre-granulosa cells, (Supplementary Fig. 1). To study its role in this crucial ovarian lineage, we generated a mouse line in which *Trim28* can be conditionally deleted using the *Nr5a1:Cre*[21,22] transgenic line (*Trim28*$^{flox/flox}$; *Nr5a1:Cre* referred as *Trim28*^{cKO} or cKO in the text/figures). In 13.5 dpc cKO ovaries, nuclear TRIM28 signal was strongly decreased in FOXL2-positive pre-granulosa cells, whereas it was still present at heterochromatin foci, and was nearly disappeared at E18.8 (Supplementary Fig. 1). At birth, XX cKO mice displayed normal external female genitalia, without any obvious ovarian structure abnormality at 3 days post-partum (dpp)(Supplementary Fig. 2). In FOXL2-positive immature granulosa cells, we did not detect any signal for TRIM28 and SOX8/SOX9, two Sertoli cell markers (Supplementary Fig. 2). Unlike granulosa cells that looked normal at this stage, oocytes were larger, suggesting an early and indirect effect of TRIM28 absence on oogenesis. This suggests that TRIM28 is not required for fetal ovary differentiation. However, as TRIM28 is still expressed in pre-granulosa cells at 13.5 dpc, a potential role in the primary ovarian determination that occurs at ~11.5 dpc cannot be excluded.

In several follicles of 20 dpp *Trim28*^{cKO} ovaries, SOX8 was expressed in groups of cells that stopped expressing FOXL2 (Fig. 1a). Some of these cells are co-expressing FOXL2 and SOX8, suggesting a transdifferentiation event. Double immunostaining showed that some SOX8-positive cells also expressed SOX9, suggesting that SOX8 expression precedes SOX9, unlike what observed in mouse embryonic testes[23]. As SOX8 and SOX9 are Sertoli cell markers, this suggests that fetal deletion of *Trim28* in pre-granulosa cells might induce their reprogramming towards Sertoli cells after birth, as described for *Foxl2* deletion[4] and oestrogen receptor double knock-out[2].

In 8-week-old *Trim28*^{cKO} mice, ovarian organisation was profoundly changed. Medullar follicles had almost completely lost FOXL2 expression, expressed SOX8 and SOX9, and were reorganised into pseudo-tubular structures, indicative of a process of testis cord formation (Supplementary Fig. 3a, d, g). We never detected any cell that expressed both SOX8 (Supplementary Fig. 3b) or SOX9 (Supplementary Fig. 3e) and FOXL2, but many cells that expressed both SOX proteins (Supplementary Fig. 3h). Their distribution suggested (like in 20 dpp *Trim28*^{cKO} ovaries) that SOX8 might precede SOX9. Conversely, the cortical region presented a less advanced phenotype: as observed in 20 dpp *Trim28*^{cKO} ovaries, follicles were still organised, but remodelling had started with groups of cells that stopped expressing FOXL2 and expressed SOX8 and/or SOX9 (Supplementary Fig. 3c, f and i). These results show that in *Trim28*^{cKO} ovaries, the granulosa-to-Sertoli cell transdifferentiation starts in follicles located in the medulla and then spread to the cortical regions.

In parallel, using the Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, we did not observe any significant increase in apoptosis in 20 dpp and 8-week-old *Trim28*^{cKO} ovaries (Supplementary Fig. 4), as previously described for the cKO of *Foxl2*[4]. This excluded the replacement by neo-formed Sertoli cells of granulosa cells eliminated by widespread apoptosis.

In 4-month-old *Trim28*^{cKO} females, the transdifferentiation of granulosa cells into Sertoli cells was complete: FOXL2 expression has disappeared, and follicles were completely remodelled into tubular structures with cells that expressed the Sertoli cell markers SOX8, SOX9 and DMRT1 (Fig. 1b). Histological analysis confirmed the progressive reorganisation of ovarian follicles into tubular structures and the transdifferentiation of granulosa cells into cells with a Sertoli cell morphology (Supplementary Fig. 5). This reorganisation was undetectable in 4-week-old *Trim28*^{cKO} ovaries but was clearly visible in the medulla at 8 weeks and was completed at 17 weeks. Germ cells (oocytes) were relatively normal in ovaries with a preserved follicular structure but started to degenerate during transdifferentiation. In 8-week-old ovaries in which the medullar part was reorganised into pseudo-tubules, oocytes had disappeared or were degenerating (Supplementary Fig. 5), and in 17-week-old ovaries they had disappeared.

A recent study showed that *Trim28* hemizygosity affects spermatogonial stem cells and induces testis degeneration[24]. However, we did not observe any change in FOXL2 immunostaining in ovaries from wild-type and heterozygous *Trim28*^{cKO} mice at the different stages we analysed (Supplementary Fig. 6a). Similarly, we did not detect any expression change of the three Sertoli markers *Sox8*, *Sox9* and *Dmrt1* in heterozygous 3-month-old ovaries (Supplementary Fig. 6b). Therefore, the loss of a single *Trim28* allele does not cause transdifferentiation of granulosa cells.

We next examined the temporal expression of several genes with roles in testicular and ovarian sex-determination in 0.5- (15 dpp), 2 and 4-month-old ovaries. Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) analysis revealed that in *Trim28*^{cKO} ovaries, the mRNA level of most ovarian-specific genes was decreased, with the exception of *Rspo1* (Fig. 1c, panel Ovarian genes). Conversely, testicular-specific genes were progressively upregulated (Fig. 1c, panel Testicular genes), confirming the histology and immunofluorescence observations. The expression level of some ovarian (*Foxl2, Esr2, Cyp19a1*, and *Rspo1*) and testicular genes (*Sox8* and *Dhh*) was already modified soon after birth (15 dpp), before changes in *Sox9* and *Dmrt1* and before the detection of histological defects (Supplementary Fig. 5).

Bulk RNA-seq experiments using 7-month-old *Trim28*^{cKO} ovaries (Data S1), in which transdifferentiation was completed, showed that 1669 genes were significantly downregulated in the absence of *Trim28*, among which 71% are normally expressed in adult granulosa cells[25], including genes involved in ovarian determination (Fig. 1d, right).