RUNX1 maintains the identity of the fetal ovary through an interplay with FOXL2

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Sex determination of the gonads begins with fate specification of gonadal supporting cells into either ovarian pre-granulosa cells or testicular Sertoli cells. This fate specification hinges on a balance of transcriptional control. Here we report that expression of the transcription factor RUNX1 is enriched in the fetal ovary in rainbow trout, turtle, mouse, goat, and human. In the mouse, RUNX1 marks the supporting cell lineage and becomes pre-granulosa cell-specific as the gonads differentiate. RUNX1 plays complementary/redundant roles with FOXL2 to maintain fetal granulosa cell identity and combined loss of RUNX1 and FOXL2 results in masculinization of fetal ovaries. At the chromatin level, RUNX1 occupancy overlaps partially with FOXL2 occupancy in the fetal ovary, suggesting that RUNX1 and FOXL2 target common sets of genes. These findings identify RUNX1, with an ovary-biased expression pattern conserved across species, as a regulator in securing the identity of ovarian-supporting cells and the ovary.
critical step that shapes the reproductive identity of the embryo is the sexual differentiation of the bipotential gonads. Supporting cells in the fetal gonads are the first cell population to differentiate and dictate the fate of the gonads. As a consequence, defects in supporting cell differentiation have dire consequences on reproductive outcomes of the individual, from sex reversal to infertility. Supporting cells differentiate into either Sertoli cells, which drive testis development, or pre-granulosa cells, which control ovarian development. It has become clear that supporting cell differentiation and maintenance of their commitment requires a coordinated action of multiple factors that play either complementary, redundant, and even antagonistic roles. For instance, fate decision and maintenance of granulosa cells, which control ovarian development. It has been shown from sex reversal to infertility. Supporting cells differentiate into somatic cell population to differentiate and dictate the fate of the gonads. Although all three RUNX transcription factors bind the same runt expression pattern implies a role in ovary development Runx1 based on transcriptomic analyses. The RUNX transcription factor critical for cell lineage determination could play a role during gonadal differentiation. Factors involved in gonad differentiation are generally conserved in vertebrates and even invertebrates, although their position in the hierarchy of the molecular cascade may change during evolution. For instance, the transcription factor FOXL2 is important for ovarian differentiation/function in human, goat, and fish. The pro-testis transcription factor DMR1 is highly conserved and critical for testis development in worms, fly, fish, and mammal.

In this study, we set up to investigate the role of transcription factor RUNX1 in the mouse fetal ovary. In Drosophila melanogaster, the RUNX ortholog runt is essential for ovarian determination. In the mouse, Runx1 mRNA is enriched in the fetal ovary based on transcriptomic analyses. The RUNX family arose early in evolution: members have been identified in metazoans from sponge to human, where they play conserved key roles in developmental processes. In vertebrates, RUNX1 acts as a transcription factor critical for cell lineage specification in multiple organs and particularly in cell populations of epithelial origin. We first characterize the expression profile of RUNX1 in the fetal gonads in multiple vertebrate species, from fish to human. We then use knockout (KO) mouse models and genomic approaches to determine the function and molecular action of RUNX1 and its interplay with another conserved ovarian regulator, FOXL2, during supporting cell differentiation in the fetal ovary.

**Results**

**Runx1 expression pattern implies a role in ovary development.**

The runt gene, critical for ovarian determination in the fly, has three orthologs in mammals: RUNXI, RUNX2, and RUNX3. Although all three RUNX transcription factors bind the same DNA motif, they are known to have distinct, tissue-specific functions. In the mouse, Runx1 was the only one with a strong expression in the fetal ovary, whereas Runx2 and Runx3 were expressed weakly in the fetal gonads in a non-sexually dimorphic way (Fig. 1a). At the onset of sex determination (Embryonic day 11.5 or E11.5), Runx1 expression was similar in both fetal XY (testis) and XX (ovary) gonads before becoming ovary-specific after E12.5 (Fig. 1b), consistent with observations by others. An ovary-enriched expression of Runx1 during the window of early gonad differentiation was also observed in other mammals such as human and goat, as well as in species belonging to other classes of vertebrates such as red-eared slider turtle and rainbow trout (Fig. 1c–f), implying an evolutionarily conserved role of RUNX1 in ovary differentiation.

To identify the cell types that express Runx1 in the gonads, we examined a reporter mouse model that produces enhanced green fluorescent protein (EGFP) under the control of Runx1 promoter. Consistent with Runx1 mRNA expression (Fig. 1b), Runx1-EGFP was present in both XX and XY gonads at E11.5, then increased in XX gonads and diminished in XY gonads at E12.5 onwards (Fig. 2). At E11.5 in both XX and XY gonads, Runx1-EGFP was present in a subset of SF1+/PECAM– somatic cell population, whereas it was absent in the SF1–/PECAM+ germ cells (Fig. 2a–d). In the XY gonads, these Runx1-EGFP+ somatic cells corresponded to Sertoli cells, as demonstrated by a complete overlap with SRY, the sex-determining factor that drives Sertoli cell differentiation. This Runx1-EGFP expression was eventually turned off in the fetal testis, while it was maintained in the ovary (Fig. 2b, i). Throughout fetal development of the ovary, Runx1-EGFP remained in FOXL2+ pre-granulosa cells (Fig. 3). Runx1-EGFP was also detected in the ovarian surface epithelium at E16.5 and birth (arrows in Fig. 3b, c), which gives rise to granulosa cells in the cortex of the ovary. Runx1-EGFP was also expressed in somatic cells of the cortical region right underneath the surface epithelium at E16.5 and some of these Runx1-EGFP+ cells presented a weak expression of FOXL2 (Fig. 3g–i, arrowheads). In summary, Runx1 marks the supporting cell lineage in the gonads at the onset of sex determination and becomes pre-granulosa cell-specific as gonads differentiate.

XX Runx1 KO and Foxl2 KO share common transcriptomic changes. Its pre-granulosa cell-specific expression suggests that Runx1, a factor involved in cell lineage determination, could contribute to granulosa cell differentiation and ovarian development. To investigate its role in gonads and avoid early embryonic lethality as a result of global deletion of Runx1, we generated a conditional KO mouse model in which Runx1 was ablated in the SF1+ gonadal somatic cells. We characterized the effects of Runx1 inactivation on XX gonad differentiation at E14.5, a stage where morphological differences between ovary and testis are already established. Although Runx1 expression was ablated successfully in XX gonads (Fig. 4d), ovarian morphogenesis appeared normal: E14.5 XX Runx1 KO gonads presented similar size and shape compared with XX control gonads (Supplementary Fig. 2), and differentiation and organization of different gonadal cell populations were similar to control XX gonads (Fig. 4a–c). For instance, XX Runx1 KO-supporting cells expressed the pre-granulosa cell marker FOXL2 but not Sertoli cell markers SOX9 and AMH (Fig. 4a, b). Supporting cells and germ-cell organization was similar to XX control gonads and did not form Laminin-outlined cord structures typical of testis differentiation (Fig. 4b). Similar to supporting cells, no difference was observed in the COUP-TFI+ interstitium organization between XX Runx1 KO and XX control gonads (Fig. 4b). Finally, E14.5 XX Runx1 KO germ cells had initiated meiosis, a typical feature of fetal ovary development, and normal expression of the germ-cell marker Mvh was observed (Fig. 4c, d). No significant change was detected for key genes involved in pre-granulosa cell
differentiation (Foxl2 and Wnt4) or Sertoli cell differentiation (Sox9, Fgf9, Nr5a1, and Amh; Fig. 4d, e). However, pre-granulosa cell marker Fst and fetal Sertoli cell marker desert hedgehog (Dhh) were mis-expressed in E14.5 XX Runx1 KO gonads (Fig. 4d, e).

Similarly, at birth, XX Runx1 KO gonads maintained a typical ovarian shape, with FOXL2+ pre-granulosa cells scattered throughout the gonad and TRA98+ germ cells located mostly in the cortex (Fig. 4f). Despite their normal ovarian morphology, newborn XX Runx1 KO gonads exhibited an aberrant transcriptomic profile reminiscent of the transcriptome of newborn XX Foxl2 KO gonads, such as downregulation of pro-ovarian genes that are direct targets of FOXL2 (Fig. 4g and Supplementary Data 1). Foxl2 is involved in ovarian differentiation/maintenance in various vertebrate species. In the mouse, loss of Foxl2 results in normal ovarian morphogenesis at birth, despite aberrant ovarian transcriptome, and eventually leads to masculinization of the ovary postnatally. We found that 41% of the genes differentially expressed in Runx1 KO were also misregulated in the absence of Foxl2 in newborn XX gonads (Fig. 4g and Supplementary Data 1).

![Diagram](https://via.placeholder.com/150)

**Fig. 1** RUNX1 expression during gonadal differentiation in various vertebrates. **a** Expression of Runx1, Runx2, and Runx3 mRNAs in XX and XY gonads of E14.5 mouse embryos (n = 5/sex). Values are presented as mean ± SEM; non-parametric t-test, **p** < 0.01; NS, not significant. **b** Expression time course of Runx1 mRNA in mouse XX and XY gonads during gonadal differentiation (n = 3/stage). Values are presented as mean ± SEM. **c-f** Time course of RUNX1 mRNA expression in four other vertebrate species, human, goat, red-eared slider turtle, and rainbow trout during gonad differentiation. Values are presented as mean ± SEM. For the turtle, pink and blue bars represent gonads at female-promoting temperature (FPT) of 31 °C and at male-promoting temperature (MPT) of 26 °C, respectively. RUNX1 expression was analyzed by RNA-seq in human and red-eared slider turtle, and by qPCR in goat and rainbow trout. Green highlighted areas represent the window of early gonadal differentiation. Source data are provided as a Source Data file.
three independent biological replicates were analyzed and the images a (PECAM-1; endogenous EGFP were co-labeled with markers for germ cells/vasculature and SOX9 in e).

Transcriptomic changes is that were fertile. One possible explanation for these common transcriptomic changes identified in Runx1 and Foxl2 KO newborn ovaries raised the question whether RUNX1 and FOXL2 could play redundant/complementary roles in supporting cell differentiation. We therefore generated Runx1/Foxl2 double KO mice (referred as DKO) and compared XX gonads differentiation in the absence of Runx1, Foxl2, or both (Fig. 5 and Supplementary Figs. 3 and 4). Abnormal development of XX DKO gonads became apparent around E15.5. At this stage, differentiation of supporting cells into Sertoli cells in the testis or pre-granulosa cells in the ovary has been established. For instance, the transcription factor DMRT1, involved in the maintenance of Sertoli cell identity, is expressed in Sertoli cells but not pre-granulosa cells (Fig. 5a, e). At E15.5, DMRT1 is also present in a few germ cells in both the testis and ovary.

Similar to control ovaries, XX gonads lacking either Runx1 or Foxl2 had no DMRT1 proteins in the supporting cells (Fig. 5a–c). However, the combined loss of Runx1 and Foxl2 resulted in aberrant expression of DMRT1 in the supporting cells of XX gonads (Fig. 5d). At birth, a time when XX Foxl2 KO gonads still morphologically resemble ovaries, XX Runx1/Foxl2 DKO gonads formed structures similar to fetal testis cords in the center, with DMRT1+ cells surrounding clusters of germ cells (Fig. 5i). Such structure was not observed in XX Runx1 KO or Foxl2 KO gonads with the exception that DMRT1 protein started to appear in a few supporting cells in the newborn XX Foxl2 KO gonads, in what appears to be one of the first signs of postnatal masculinization of Foxl2 KO ovaries at the protein level (Fig. 5h).

Contrary to DMRT1, SOX9 protein, a key driver of Sertoli cell differentiation, was not detected in XX DKO newborn gonads (Fig. 6). Our results demonstrate that a combined loss of Runx1/Foxl2 induces partial masculinization of the supporting cells during fetal development of the ovary.

Runx1 and Foxl2 double knockout results in partial masculinization of fetal ovaries. The common transcriptomic changes identified in Runx1 and Foxl2 KO newborn ovaries raised the question whether RUNX1 and FOXL2 could play redundant/complementary roles in supporting cell differentiation. We therefore generated Runx1/Foxl2 double KO mice (referred as DKO) and compared XX gonads differentiation in the absence of Runx1, Foxl2, or both (Fig. 5 and Supplementary Figs. 3 and 4). Abnormal development of XX DKO gonads became apparent around E15.5. At this stage, differentiation of supporting cells into Sertoli cells in the testis or pre-granulosa cells in the ovary has been established. For instance, the transcription factor DMRT1, involved in the maintenance of Sertoli cell identity, is expressed in Sertoli cells but not pre-granulosa cells (Fig. 5a, e). At E15.5, DMRT1 is also present in a few germ cells in both the testis and ovary.

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To further characterize the impacts of the combined loss of Runx1/Foxl2 on ovarian differentiation, we compared the transcriptome of newborn XX Runx1/Foxl2 DKO gonads with the transcriptomes of XX control, Runx1, or Foxl2 single KO gonads (Fig. 7 and Supplementary Data 2). The heat map for the 918 differentially expressed genes between XX Runx1/Foxl2 DKO and XX control gonads demonstrated allele-specific impacts: loss of Runx1 resulted in a mild and often nonsignificant effect on these genes, loss of Foxl2 had an intermediate/strong effect, and combined loss of Runx1/Foxl2 yielded the strongest effect (fold change > 1.5; p < 0.05 one-way analysis of variance (ANOVA); Fig. 7a and Supplementary Data 3). Gene ontology analysis revealed that the downregulated genes were associated with “ovarian follicle development” and “female gonad development,” whereas “male sex determination” was the most significantly enriched process for the upregulated genes (Supplementary Fig. 5). Conforming to the hierarchical clustering (Fig. 7a), comparison of the genes significantly changed in Runx1/Foxl2 single and DKO suggested that Foxl2 was the main contributor to the transcriptional changes observed in Runx1/Foxl2 DKO (Fig. 7b–g and Supplementary Data 4): 61% of the genes downregulated in DKO were also downregulated in Foxl2 KO and 43% of the genes upregulated in DKO were also upregulated in Foxl2 KO. In addition, some genes appeared to be controlled by both Foxl2 and Runx1, and were significantly downregulated or upregulated in all three KOs (Fig. 7b, c). For instance, the genes Fst and Cyp19a, both involved in granulosa cell differentiation/function, were downregulated in Runx1 KO, but their expression was not affected by Foxl2.

**Fig. 2** Runx1 is expressed in the supporting cells during gonad differentiation. a–g Whole-mount immunofluorescence of XY and XX gonads from Tg(Runx1-EGFP) reporter mice at E11.5 and E12.5. Gonads with endogenous EGFP were co-labeled with markers for germ cells/vasculature (PECAM-1; a, b), somatic cells (SF1; c, d), Sertoli cells in XY gonads (SRY in e and SOX9 in f), and for pre-granulosa cells in XX gonads (FOXL2; g). Scale bars: 100 µm. Single-channel images are provided for e, f in Supplementary Fig. 1. h, i Detection of endogenous EGFP in freshly collected E14.5 gonads. Scale bars: 200 µm. Dotted lines outline the gonads. At least three independent biological replicates were analyzed and the images presented are representative of all replicates.
Foxl2 KO, and more repressed in Runx1/Foxl2 DKO (Fig. 7d). On the other hand, Dhh was upregulated in all three KOs, with the highest expression in the DKO (Fig. 7f). Finally, some genes were significantly changed in Runx1/Foxl2 DKO only, suggesting a cumulative effect of Runx1 and Foxl2 loss. For instance, Foxp1, a gene whose expression is enriched in pre-granulosa cells40, was significantly downregulated only in Runx1/Foxl2 DKO (Fig. 7e), whereas Fgf9, a Sertoli gene contributing to testis differentiation41, and Pdgfc were significantly upregulated only in Runx1/Foxl2 DKO (Fig. 7g).

In contrast to XX Foxl2 single KO gonads (Fig. 5), in which sex reversal only became apparent postnatally8, XX Runx1/Foxl2 DKO gonads exhibited masculinization with visible morphological changes before birth. To determine how the additional loss of Runx1 contributed to the earlier masculinization of Runx1/Foxl2 DKO ovaries, we identified the genes differentially expressed between XX Runx1/Foxl2 DKO and XX Foxl2 KO gonads (Fig. 7h and Supplementary Data 5). Expression of most of these genes was already altered in XX Foxl2 single KO gonads; however, the additional loss of Runx1 exacerbated their mis-expression. For instance, the pro-testis gene Dmrt1 and Nr5a1 were significantly upregulated, whereas the pre-granulosa-cell-enriched transcripts Fst and Ryr2 were further downregulated at birth (Fig. 7d, i). On the other hand, the additional loss of Runx1 did not cause further upregulation of the Sertoli genes Sox9 and Amh at birth, suggesting that Runx1 does not contribute to their repression in the ovary (Fig. 7i). Overall, the transcriptomic analyses of Runx1/Foxl2 single and DKO gonads revealed that Foxl2 is the main driver of the transcriptomic changes, and that the additional loss of Runx1 amplifies the mis-expression of genes already altered by the sole loss of Foxl2, leading to the failure to maintain pre-granulosa cell identity in the fetal ovary.

RUNX1 shares genome-wide chromatin occupancy with FOXL2. The masculinization of XX Runx1/Foxl2 DKO fetal gonads and the transcriptomic comparisons of newborn XX Runx1/Foxl2 single and DKO gonads suggest some interplay
between RUNX1 and FOXL2 to control pre-granulosa cell identity. The fact that RUNX1 and FOXL2 are both transcription factors expressed in the pre-granulosa cells (Fig. 3 and Supplementary Fig. 1) raised the question whether this interplay could occur directly at the chromatin level. We have previously identified FOXL2 chromatin occupancy during ovarian differentiation by chromatin immunoprecipitation (ChIP) followed by whole-genome sequencing at E14.5, a time where FOXL2 and RUNX1 expression fully overlaps (Fig. 3). We performed additional de novo motif analyses on the genomic regions bound by FOXL2 in the fetal ovary, and discovered that several other DNA motifs were co-enriched with FOXL2 DNA motif (Fig. 8a).
motif was the second most significantly co-enriched motif. The other motifs were for CTCF, a factor involved in transcriptional regulation, enhancer insulation, and chromatin architecture\(^43\), and for the DNA motif recognized by members of the nuclear receptor family including liver receptor homolog-1 (LRH-1 encoded by \(Nr5a1\)) and SF1 (encoded by \(Nr5a1\)), a known co-factor of FOXL2\(^44,45\). DNA motifs for TEAD transcription factors involved in gonad differentiation\(^46\), were also significantly enriched. The enrichment of RUNX motif with FOXL2-binding motif suggests that RUNX1, the only RUNX also expressed in pre-granulosa cells, could bind similar genomic regions to FOXL2 in the fetal ovary. To confirm this hypothesis, we performed ChIP-sequencing (ChIP-seq) for RUNX1 in E14.5 ovaries (Supplementary Data 6), the same stage as FOXL2 ChIP-seq\(^42\). The top de novo motif identified in RUNX1 ChIP-seq \((p<1e−559)\) matched the RUNX motif\(^47\) (Fig. 8b) and corresponded to the motif that was co-enriched with FOXL2 in FOXL2 ChIP-seq (Fig. 8a). A total of 10,494 RUNX1-binding peaks were identified in the fetal ovary, with the majority of the peaks located either in the gene body (Fig. 8c; 25% exon and 22% intron) or close upstream of the transcription start site (TSS) (30% Promoter: < 1 kb of TSS; 12% Upstream: −10 to −1 kb of TSS). Comparison of genome-wide chromatin binding of RUNX1 and FOXL2 in the fetal ovary revealed significant overlap: 54% (5619/10,494) of RUNX1 peaks overlapped with FOXL2 peaks (Fig. 8d).

The transcriptomic data from \(Runx1/Foxl2\) DKO ovaries provided us a list of genes significantly changed as a result of the absence of \(Runx1, Foxl2\), or both (Fig. 7). To identify potential direct target genes of RUNX1 or/and FOXL2, we focused on the 918 genes differentially expressed in \(Runx1/Foxl2\) DKO ovaries and determined which genes were nearest to RUNX1 or/and FOXL2-binding peaks (Fig. 9a and Supplementary Data 7). More than 50% of these genes (492/918; Fig. 9a) were the closest gene to RUNX1 or/and FOXL2 peaks. Some of these genes were nearest to only FOXL2 peaks (116 genes in Fig. 9a). For example, \(Pla2r1\), a transcript enriched in pre-granulosa cells\(^46\), was strongly downregulated in both \(Foxl2\) KO and \(Runx1/Foxl2\) DKO fetal ovaries (Fig. 9b), contained two FOXL2-specific peaks, one in the promoter and one in the first intron. On the other hand, 102 genes (Fig. 9a) had RUNX1-specific peaks near their genomic locations. For instance, \(Ryr2\), another transcript enriched in pre-granulosa cells\(^40\), was strongly downregulated in both \(Runx1\) KO and \(Runx1/Foxl2\) DKO fetal and newborn ovaries (Figs. 7i and 9c), and contained one RUNX1-specific peak in its intronic region. Finally, 274 genes were the closest genes to the peaks for both RUNX1 and FOXL2, with the majority of them (197 genes) nearest to overlapping peaks for RUNX1 and FOXL2 (Fig. 9a). Most of these genes were downregulated in \(Runx1/Foxl2\) DKO ovaries (Supplementary Data 7). For instance, the pre-granulosa cell-enriched genes \(Ps1\) and \(Itpr2\), both downregulated in \(Runx1/Foxl2\) single and DKO ovaries (Figs. 7d and 9d), contained common binding peaks for FOXL2 and RUNX1 (Fig. 9a, d). For \(Ps1\), this binding of RUNX1 and FOXL2 was located in its first intron, in the previously identified regulatory region that contributes to its expression\(^42,48\). On the other hand, Sertoli cell -enriched gene \(Dmrt1\), which was strongly upregulated in \(Runx1/Foxl2\) DKO (Figs. 5 and 7i), contained a common binding site for FOXL2 and RUNX1 near its promoter (Fig. 9a). Taken together, our results reveal that RUNX1, a transcription factor expressed in

| FOXL2 | TRA98 | DMRT1 |
|-------|-------|-------|
| XX Control | XX Runx1 KO | XX Foxl2 KO |
| XX Runx1/Foxl2 KO | XY Control |

![Fig. 5 Combined loss of Runx1 and Foxl2 masculinizes the fetal ovaries.](https://example.com/fig5)

The table above shows the experimental conditions used in the experiment. The top panel of the figure (a-e) shows the results of the experiment at E15.5, and the bottom panel (f-j) shows the results at birth (P0). The controls correspond to wild-type littermates. The gray represents DAPI nuclear staining. Dotted lines outline the gonads. Higher magnifications are shown for the outlined boxes in (a-e) and (f-j), respectively. Scale bars: 100 μm. At least three independent biological replicates were analyzed and the images presented are representative of all replicates. Single-channel images are provided in Supplementary Figs. 3 and 4.
Runx1 development. In contrast, combined loss of scriptome but did not affect ovarian morphogenesis during fetal somatic cells of the ovaries altered ovarian trans-morphogenesis29,36,49. This is not the case in the mouse ovary, of-function mouse models for SRY or SOX9, the two transcrip-tomic analyses, we propose that FOXL2 is the dominant player and RUNX1 acts as a supporting player. For instance, global KO of Foxl2 in XX gonads results in postnatal sex reversal16, whereas the XX Runx1 single KO has no such impacts. In addition, loss of Foxl2 leads to transcriptomic changes more promi-nent than loss of Runx1 in newborn ovaries, even though they affect common sets of genes. However, the additional loss of Runx1 amplifies the transcriptomic changes caused by the loss of Foxl2, resulting in fetal masculinization. One of the most striking changes in Runx1/Foxl2 DKO ovaries is the expression of DMRT1 in the fetal supporting cells. DMRT1 is a key driver of Sertoli cell differentiation and testis development in various species16,21. In the fly, doublesex (dsx), an ortholog of mammalian DMRT1, controls testis differentiation18. Intriguingly, runt, the fly ortholog of RUNX1, tips the balance toward ovarian determination by antagonizing the testis-specific transcriptional regulation of dsx22. In the mouse, testis differentiation is not controlled by DMRT1 but by SOX transcription factor SRY and its direct target SOX9. However, RUNX1 does not appear to contribute to the repression of the key pro-testis gene Sox9 in the fetal ovary and SOX9 protein was not detected in Runx1/Foxl2 DKO ovary at birth. This is in contrast with the phenotype of Wnt4/Foxl2 DKO newborn ovaries where SOX9 was upregulated, and as a consequence the ovaries were more masculinized10. Overall, our findings suggest that slightly different pro-ovarian networks control the repression of the evolutionary conserved pro-testis genes Sox9 and Dmrt1: Sox9, which plays a primary role in Sertoli cell differentiation in the mouse, is repressed by an interplay between the WNT4/RSPO1/β-catenin and FOXL29,10. On the other hand, Dmrt1, which has taken a secondary role in Sertoli cell differentiation in the mouse, is repressed by an interplay between RUNX1 and FOXL2. The fact that RUNX1 does not appear to control Sox9 may be the reason why RUNX1 only plays a secondary role in granulosa cell differentiation/maintenance, in contrast to the dominant roles of the WNT pathway and FOXL2. It would be interesting to determine the role of RUNX1 in species for which DMRT1 plays a more prominent role in initiation of differentiation of the testis.

Seeking the mechanisms underlying the interplay between RUNX1 and FOXL2 in the regulation of pre-granulosa cell identity, we identified that RUNX DNA-binding motif is sig-nificantly co-enriched with FOXL2 motif in genomic regions bound by FOXL2 in the fetal ovary. The fact that RUNX1 genome-wide chromatin occupancy partially overlaps with FOXL2 in the fetal ovary, that RUNX1 and FOXL2 are expressed in the same cells at the time of the ChIP-seqs, that their loss affects common set of genes, and that the DKO results in gonad masculinization, altogether support the model in which RUNX1 and FOXL2 jointly occupy common chromatin regions that control the maintenance of pre-granulosa cell identity. By themselves, RUNX proteins are weak transcription factors and they require other transcriptional regulators to function as either repressors or activators of transcription26. Interplay between RUNX1 and several members of the forkhead transcription factor family has been documented in different tissues. For instance, RUNX1 is a co-activator of FOXO3 in hepatic cells50. Similarly, an interplay between RUNX1 and FOXO1/FOXO3 was demonstrated in breast epithelial cells where a subset of FOXO target genes were jointly regulated with RUNX151. Another forkhead protein, FOXP3, acts with RUNX1 to control gene expression in T cells52 and breast epithelial cells53. Such cooperation in various tissues suggest that the interplay between RUNXs and forkhead transcription factors maybe an evolutionary conserved phenomenon. It would be interesting to determine whether the forkhead protein FOXL2 is also able to physically interact with RUNX1 in the fetal ovaries and how this interaction occurs. Our data and single-cell sequencing data27 demonstrate that RUNX1 starts to...
be expressed in the supporting cells earlier than FOXL2. In other tissues, it was demonstrated that RUNX1 can function as a pioneer factor that allows chromatin remodeling and recruitment of other factors to control gene expression and cell fate. Therefore, it is possible that RUNX1 is first recruited to chromatin regions and the presence of RUNX1 facilitates the recruitment of FOXL2 to maintain pre-granulosa cell identity. Finally, in addition to the genes co-regulated by FOXL2 and RUNX1, we identified genes that were specifically mis-expressed in the absence of RUNX1 but not FOXL2. Genome-wide analyses of RUNX1 binding in the fetal ovary also identified genomic regions bound by RUNX1 but not FOXL2. These results suggest that RUNX1 could also contribute to ovarian development or function independently of FOXL2.

RUNX1 contributes to cell-fate determination in various developmental processes such as hematopoiesis and hair follicle development. Depending on its interplay with other signal transduction pathways or co-factors, RUNX1 controls which path the precursor cells take when they are at the crossroad between cell proliferation/renewal and lineage-specific commitment. We discovered that RUNX1 has an ovary-biased expression during gonad differentiation in various vertebrate species, including turtle, rainbow trout, goat, mouse, and human. In mouse...
embryonic gonads, Runx1 is first detected in the supporting cells in a non-sexually dimorphic way at the onset of sex determination. Although its expression is maintained in the ovary, Runx1 appears to be actively repressed in the testis between E11.5 and E12.5 as the supporting cells commit to Sertoli cell fate. The suppression of Runx1 in the fetal testis is corroborated by previously published data from a time-course transcriptomic analysis during early gonad development55 and single-cell sequencing analysis of SF1+ progenitor cells27,56. The time course of Sertoli cell differentiation at the single-cell level revealed that Runx1 follows a similar spatiotemporal pattern of expression with Sry56.

In the mouse, Sry expression in Sertoli cells is quickly turned off after the initiation of testis differentiation and it is suspected that the repression of Sry is due to a negative feedback loop by downstream pro-testis genes. The similar pattern of down-regulation of Runx1 in the testis after E11.5 raises the possibility that Runx1 is downregulated by a similar signaling pathway. Regulation of Runx1 gene expression is complex and several enhancers that confer tissue-specific expression have been identified57. It remains to be determined how Runx1 expression is controlled in the gonads and how it is actively repressed in the fetal testis.

In contrary to the testes, fetal ovaries maintain expression of Runx1 in the supporting cells as they differentiate into pre-granulosa cells. During ovarian differentiation, granulosa cells arise from two different waves: the first cohort of granulosa cells arises from the bipotential supporting cell precursors that differentiate into either Sertoli cells or pre-granulosa cells during sex determination58. The second wave of granulosa cells that eventually populate the cortical region of the ovary appears later in gestation. This second wave arises from LGR5+ cells of the ovarian surface epithelium that ingress into the ovary from E15.5 to postnatal day 4 and eventually become LGR5-/FOXL2+ granulosa cells30,31. This timing of establishment of the second cohort of granulosa cells correlates with the expression of Runx1-EGFP in a subset of cells in the surface epithelium and in granulosa cells of the ovarian cortex at E16.5 and birth. These results suggest that Runx1 also marks granulosa cell precursors that will give rise to the second wave of FOXL2+ granulosa cells in the cortex. Therefore, both expression at onset of sex determination and at the surface epithelium/cortex at the time of the second wave of granulosa cells recruitment suggest that Runx1 is activated in cells that are primed to become supporting/granulosa cells.

Fig. 8 RUNX1 and FOXL2 exhibit overlaps in chromatin binding in fetal ovaries. a de novo motif analysis of FOXL2 peaks identifies enrichment of RUNX motif along with FOXL2 motif in E14.5 ovaries. b The top de novo motif for RUNX1 ChIP-seq in E14.5 ovaries corresponds to a RUNX motif. c Distribution of genomic location of the 10,494 RUNX1-binding peaks. TSS, transcription start site; TES, transcription end site. d Comparison of RUNX1 (10,494 peaks) and FOXL2 (11,438 peaks) chromatin occupancy in E14.5 ovaries.
Multiple transcription factors often form complex genetic regulatory networks that control cell-fate determination. Genomic sequence motifs or cis-regulatory elements for the supporting cell lineage in the testis were identified by combined analyses of SOX9 and DMRT1 ChIP-seq, and by motif prediction. These “Sertoli cell signatures” are composed of binding motifs for transcription factors critical for Sertoli cell differentiation, including SOX9, GATA4, and DMRT1. These Sertoli cell signatures, present in mammals and other vertebrates, could represent a conserved regulatory code that governs the cascade of Sertoli cell differentiation, regardless of whether it primarily relies on SOX transcription factors critical for Sertoli cell differentiation, regardless of whether it primarily relies on SOX transcription factors of the TEAD family belong to the Hippo pathway, which is involved in the regulation of Sertoli cell gene expression. TEADs and GATAs were also significantly enriched in SOX transcription factors critical for Sertoli cell differentiation, regardless of whether it primarily relies on SOX transcription factors such as SRY in mammals or on DMRT1 such as in several vertebrate species. Similarly, one would expect the presence of conserved “granulosa cell signature” genomic regions that confers granulosa cell differentiation. As FOXL2 is a highly conserved gene in granulosa cell differentiation in vertebrates, we used FOXL2 as an anchor factor to identify other factors that could take part in the regulatory network controlling granulosa cell differentiation/function. Unbiased analyses of the motifs co-enriched with FOXL2 motif in the fetal ovary identified the RUNX motif as one of the most co-enriched motifs. In addition to the RUNX motif, motifs for CTCF, nuclear receptors SF1/LRH-1/ESRRB, and transcription factors TEADs and GATAs were also significantly enriched with FOXL2 consensus motif in FOXL2-bound chromatin regions. For many of these transcription factors, their potential role in gonad differentiation is unknown or limited. For example, the transcription factors of the TEAD family belong to the Hippo pathway, which is involved in the regulation of Sertoli cell gene expression in the fetal gonads. However, the potential involvement of the hippo pathway in granulosa cell differentiation has not been investigated.

**Fig. 9** Identification of potential direct target genes for RUNX1 and/or FOXL2. a Pie-chart identifying the genes significantly changed in XX Runx1/Foxl2 DKO gonads that are nearest to peaks for FOXL2 and/or RUNX1. Genome browser view of two key genes significantly changed in XX Runx1/Foxl2 DKO gonads and bound by RUNX1 and FOXL2 in E14.5 ovaries. b-d Examples of genes affected in XX Runx1/Foxl2 DKO gonads and bound by FOXL2 or/and RUNX1. For each gene, we show the genome browser view of RUNX1 and/or FOXL2 binding in E14.5 ovaries, the gene expression by quantitative PCR in XX Runx1/Foxl2 single and double knockouts gonads at E15.5. Source data are provided as a Source Data file.
In conclusion, we identified RUNX1 as a transcription factor involved in pre-granulosa cell differentiation/maintenance. RUNX1 first delineates the supporting cell lineage and then becomes pre-granulosa cell-specific during gonadal development. RUNX1 plays redundant roles with FOXL2 through binding of common chromatin regions and control of common sets of genes to maintain pre-granulosa cell identity in the fetal ovary. Our findings provide insights into the genomic control of granulosa cell differentiation and pave the way for the identification of transcription factors and cis-signatures contributing to the fate determination of granulosa cells and the consequent formation of a functional ovary.

**Methods**

**Mouse models.** Tg(Runx1-1EGFP) reporter mouse was purchased from MMRRC (MMRRC_M01371-UCD) and CD-1 mice were purchased from Charles River (stock no. 131605, Runx1fl/fl (B6.129S-Ruxn1tm1Sf1jt) and Runx1fl/fl (B6.129P2-Runx1Immpf)) mice were purchased from the Jackson Laboratory (stock numbers 005669 and 008772, respectively). Sft-CreTg18 mice14 (B6D2-Tg(Nr5a1-cre)1Klp) were provided by Dr. Keith Parker and Fox2fl/fl mice16 (B6;129-Sgk1cre < tdfmrpl > ) were provided by Dr. David Schlesinger (National Institute on Aging, U.S. National Institutes of Health). Foxl2fl/fl; Runx1fl/fl mice were generated by mating Foxl2fl/fl females with male mice overnight and the females were checked for the presence of vaginal plug the next morning. The day when the vaginal plug was detected was considered embryonic day 0.5. All experiments were performed on at least two animals for each genotype and a minimum of three biological replicates per group were performed by the National Institutes of Health Animal Care and Use Committee, and were performed in accordance with an approved National Institute of Environmental Health Sciences animal study proposal.

**Immunofluorescences.** For the Tg(Runx1-1EGFP) mice, gonads were collected and fixed in 4% paraformaldehyde for 1–2 h at room temperature. Immunofluorescence experiments were performed on whole gonads at E11.5 and E12.5, and on sections for E14.5 and E15.5. All samples were processed and stained with 20 μg/ml (mouse) or 6 μg/ml (rabbit) antibodies (see below). All animal procedures were approved by the Institutional Animal Care and Use Committee. All samples were blocked in blocking buffer (5% donkey serum/0.1% Triton X-100 in phosphate-buffered saline (PBS)) for 1 h at room temperature. The samples were then incubated overnight at 4 °C in the primary antibodies diluted in blocking buffer. The next day, the samples were washed three times in 0.1% Triton X-100 in PBS and were incubated for 1 h at room temperature in the secondary antibodies diluted in blocking buffer. The samples were then washed and counterstained with DAPI (4′,6-diamidino-2-phenylindole). The antibodies used in this study are listed in Supplementary Table 1. Whole mount gonads by direct fluorescent imaging and an anti-GFP antibody was used for immunofluorescences on sections. For the different KO models, gonads were fixed in 4% paraformaldehyde overnight at 4 °C and immunofluorescence experiments were performed on paraffin sections of E11.5, E12.5, and P0 gonads as previously described15. Briefly, the samples were dehydrated and citrate-based antigen retrieval protocol was performed. Subsequently, the samples were blocked in blocking buffer (5% goat serum/0.3% Triton X-100 in phosphate-buffered saline (PBS)) for 1 h at room temperature. The samples were then incubated overnight at 4 °C in the primary antibodies diluted in blocking buffer. The next day, the samples were washed three times in 0.1% Triton X-100 in PBS and were incubated for 1 h at room temperature in the secondary antibodies diluted in blocking buffer. The samples were then washed and counterstained with DAPI (4′,6-diamidino-2-phenylindole). The antibodies used in this study are listed in Supplementary Table 1. Whole gonads and sections were imaged under a Leica DMi4000 confocal microscope. For all immunofluorescence experiments, at least three independent biological replicates were analyzed and the image presented in the figures were representative of all replicates.

**Real-time PCR analysis in the mouse.** For the time-course kinetics of Runx1 expression, fetal gonads from CD-1 embryos at embryonic day E11.5, E12.5, E13.5, E14.5, E15.5, and postnatal day P3 were separated from the mesonephros and snap-frozen. For each stage, three biological replicates were collected, with six gonads/replicate for the E11.5 stage and three gonads/replicate for the other stages. For Runx1 KO analysis, control and KO ovaries were collected at E14.5 (n = 9, three biological replicates/genotype). For Runx1/Fox1 KO/DKO analysis, control, Runx1f/f, Fox2f/f, and Fox2f/f single and DKO ovaries were collected at E15.5 (n = 4–5 genotype) and P0 (n = 4–5 genotype). For all experiments, total RNA was isolated for each replicate using RNAzol® (InVitrogen Corp., Carlsbad, CA). Gene expression was analyzed by real-time PCR using Bio-Rad CFX96TM Real-Time PCR Detection System. Gene expression was normalized to Gapdh. The Taqman probes and primers used to detect transcript expression are listed in Supplementary Tables 2 and 3. Data were analyzed using Prism GraphPad Software by unpaired Student’s t-test or by ANOVA p < 0.05. Values are presented as mean ± SEM.

**Runx1 expression in other species.** For the rainbow trout, Runx1 expression during gonadal development was assessed by quantitative PCR36. Species-specific primers were used for each species in Supplementary Table 3 for trout. Foxl2 KO expression during gonadal development was assessed at Female-Promoting Temperature of 31 °C and at Male-Promoting Temperature of 26 °C by RNA-seq analysis (RNA-sequencing). For the goat, Runx1 expression during gonadal development was assessed by quantitative PCR and two to three biological replicates were used for each stage of development. Values are presented as mean ± SE. All goat-handling procedures were conducted in compliance with the guidelines on the Care and Use of Agricultural Animals in Agricultural Research and Teaching in France (Authorization number 91–649 for the Principal Investigator, and national bioethics panel for all investigators, www.A3000524.fr). For the human, Runx1 expression during gonadal development was assessed by RNA-seq. Human fetuses (6–12 GW) were obtained from legally induced normally progressing terminations of pregnancy performed in Rennes University Hospital in France. Tissues were collected with women’s written consent, in accordance with the legal procedure agreed by the National agency for biomedical research and the local ethical committee of Rennes University Hospital in France (advice # 11–48).

**Microarray analysis.** Gene expression analysis of control, Runx1 KO, Fox2 KO, and Runx1/Fox2 DKO ovaries was conducted using Affymetrix Mouse Genome 430 2.0 GeneChip® arrays (Affymetrix, Santa Clara, CA) on four biological replicates per genotype and per replicate. Full length RNA was fragmented and labeled as directed in the WT-Ovation Pico RNA Amplification System and Encore Biotin Module protocols. Amplified biotin-aRNA (4.6 µg) was fragmented and hybridized to each array for 18 h at 45 °C in a rotating hybridization array. Slide arrays were stained with streptavidin-phycocerythrin utilizing a double-antibody staining procedure and then washed for antibody amplification according to the GeneChip Hybridization, Wash and Stain Kit, and user manual following protocol FS450-0004. Arrays were scanned in an Affymetrix scanner 3000 and data were obtained using the GeneChip® Command Console Software (AGCC, Version 3.2) and Expression Console (Version 1.2). Microarray data have been deposited in GEO under accession code GSE129038. Gene expression analyses were conducted with Partek software (St. Louis, Missouri) using a one-way ANOVA comparing the Robust Multichip Average (RMA)-normalized log2 intensities. A full dataset Excel file containing the normalized log2 intensity of all genes for each genotype and a graphic view of their expression is provided in Supplementary Data 2. In order to identify differentially expressed genes, ANOVA was performed to determine whether there was a statistical difference between the means of groups and the gene lists were filtered with p < 0.05 and fold-change cutoff of 1.5. The heat map was created comparing the genes that were significantly different between control and Runx1/Fox2 DKO ovaries. Venn diagrams were generated in Partek by comparing gene symbols between the lists of genes differentially expressed.

**Chip-seq assays and analysis.** Ovaries from E14.5 CD-1 embryos were separated from the mesonephros, snap-frozen, and stored at −80 °C. RUNX1 ChIP-seq experiments and analyses in E14.5 ovaries were performed using the same protocol than FOX229 ChIP-seq52. Two independent ChIP-seq experiments were performed for each of the included genotypes (one P0 gonad per replicate) for each genotype. For the red-eared slider turtle, ChIP-seq experiments and analyses in E14.5 ovaries were performed using the same protocol as for mammalian species. The experimental design was based on the following protocol FS450-0004. Arrays were scanned in an Affymetrix scanner 3000 and data were obtained using the GeneChip® Command Console Software (AGCC, Version 3.2) and Expression Console (Version 1.2). Microarray data have been deposited in GEO under accession code GSE129038. Gene expression analyses were conducted with Partek software (St. Louis, Missouri) using a one-way ANOVA comparing the Robust Multichip Average (RMA)-normalized log2 intensities. A full dataset Excel file containing the normalized log2 intensity of all genes for each genotype and a graphic view of their expression is provided in Supplementary Data 2. In order to identify differentially expressed genes, ANOVA was performed to determine whether there was a statistical difference between the means of groups and the gene lists were filtered with p < 0.05 and fold-change cutoff of 1.5. The heat map was created comparing the genes that were significantly different between control and Runx1/Fox2 DKO ovaries. Venn diagrams were generated in Partek by comparing gene symbols between the lists of genes differentially expressed.

Data availability

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author.
upon reasonable request. Microarray and ChIP-seq data generated in this study have been deposited in the GEO database under accession codes GSE129038 and GSE128767, respectively. The ChIP-seq data are available in the ReproGenomics Viewer (https://rgv. genouest.org). Raw data underlying all reported mean values in graphs are provided in the Source Data File. All other relevant data supporting the key findings of this study are available in the Supplementary Information files. The source data underlying Figs. 1, 4d–e, 4h, 7d–g, 7i–j, and 9b–d are provided as a Source Data file.

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**Authors contributions**

B.N. performed the experiments in the mouse. B.N. and H.H.-C.Y. designed the study, analyzed data, and wrote the paper. S.A.G performed bioinformatic analyses. F.C. and E.L. analyzed RUNX1 expression in human fetal gonads. M.P. and E.P. analyzed RUNX1 expression in the goat. E.D.-D. and Y.G. analyzed runx1 expression in rainbow trout. B.C. analyzed Runx1 expression in the red-eared slider turtle. S.A.G., E.L., M.P., E.P., E.D.-D., Y.G., B.C. and H.H.-C.Y. edited the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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