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1 **RUNX1 safeguards the identity of the fetal ovary through an interplay with FOXL2**

2

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18

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20 ovary, cell fate, disorders of sex development, sex reversal

21

22

23 **Abstract**

24 Sex determination of the gonads begins with fate specification of gonadal supporting  
25 cells into either ovarian granulosa cells or testicular Sertoli cells. This process of fate  
26 specification hinges on a balance of transcriptional control. We discovered that  
27 expression of the transcription factor RUNX1 is enriched in the fetal ovary in rainbow  
28 trout, turtle, mouse, goat and human. In the mouse, RUNX1 marks the supporting cell  
29 lineage and becomes granulosa cell-specific as the gonads differentiate. RUNX1 plays  
30 complementary/redundant roles with FOXL2 to maintain fetal granulosa cell identity,  
31 and combined loss of RUNX1 and FOXL2 results in masculinization of the fetal ovaries.  
32 At the chromatin level, RUNX1 occupancy overlaps partially with FOXL2 occupancy in  
33 the fetal ovary, suggesting that RUNX1 and FOXL2 target a common set of genes.  
34 These findings identify RUNX1, with an ovary-biased pattern conserved across species,  
35 as a novel regulator in securing the identity of ovarian supporting cells and the ovary.  
36

37           A critical step that shapes the reproductive identity of the embryo is the sexual  
38 differentiation of the bipotential gonads. Supporting cells in the fetal gonads are the first  
39 cell population to differentiate, and dictate the fate of the gonads. As a consequence,  
40 defects in supporting cell differentiation have dire consequences on reproductive  
41 outcomes of the individual, from sex-reversal to infertility. Supporting cells differentiate  
42 into either Sertoli cells, which drive testis development, or granulosa cells, which control  
43 ovarian development. It has become clear that supporting cell differentiation, and  
44 maintenance of their commitment, requires a coordinated action of multiple factors that  
45 play either complementary, redundant and even antagonistic roles<sup>1</sup>. For instance, fate  
46 decision and maintenance of ovarian identity relies mainly on two conserved elements:  
47 the WNT4/RSPO1/beta-catenin pathway<sup>2, 3, 4</sup> and the transcription factor FOXL2<sup>5, 6, 7</sup>.  
48 These two elements synergistically promote expression of pro-ovarian genes and at the  
49 same time, antagonize key pro-testis factors such as SOX9 and DMRT1. However, the  
50 combined loss of these two key pro-ovarian signaling only results in an incomplete  
51 inactivation of ovarian differentiation, suggesting that additional pro-ovarian factors are  
52 at play during gonadal differentiation<sup>8, 9</sup>. Factors involved in gonad differentiation are  
53 generally conserved in vertebrates and even invertebrates, although their position in the  
54 hierarchy of the molecular cascade may change during evolution<sup>10</sup>. For instance, the  
55 pro-ovarian transcription factor FOXL2 is important for ovarian differentiation/function in  
56 human<sup>11</sup>, goat<sup>12</sup> and fish<sup>13, 14</sup>. The pro-testis transcription factor DMRT1 is highly  
57 conserved and critical for testis development in worms, fly<sup>15</sup>, fish<sup>16, 17</sup> and mammals<sup>18, 19,</sup>  
58 <sup>20</sup>.

59           In this study, we set up to investigate the role of transcription factor RUNX1 in the  
60 mouse fetal ovary. In *Drosophila melanogaster*, the *RUNX* ortholog *runt* is essential for  
61 ovarian determination<sup>21, 22</sup>. In the mouse, *Runx1* mRNA is enriched in the fetal ovary  
62 based on transcriptomic analyses<sup>23</sup>. The RUNX family arose early in evolution<sup>24</sup>:  
63 members have been identified in metazoans from sponge to human, where they play  
64 conserved key roles in developmental processes. In vertebrates, RUNX1 acts as a  
65 transcription factor critical for cell lineage specification in multiple organs, and  
66 particularly in cell populations of epithelial origin<sup>25</sup>. We first established the expression  
67 profile of *RUNX1* in the fetal gonads in multiple vertebrate species from fish to human.  
68 We then used knockout mouse models and genomic approaches to determine the  
69 function and molecular action of RUNX1 and its interplay with another conserved  
70 ovarian regulator, FOXL2, during supporting cell differentiation in the fetal ovary.  
71

## 72 **Results**

### 73 **The pattern of *Runx1* expression implies a potential role in fetal ovarian** 74 **differentiation**

75 The *runt* gene, critical for ovarian differentiation in the fly<sup>21</sup>, has 3 orthologs in  
76 mammals: *RUNX1*, *RUNX2* and *RUNX3*. While all three RUNX transcription factors  
77 bind the same DNA motif, they are known to have distinct, tissue-specific functions<sup>26</sup>. In  
78 the mouse, *Runx1* was the only one with a strong expression in the fetal ovary whereas  
79 *Runx2* and *Runx3* were expressed weakly in the fetal gonads in a non-sexually  
80 dimorphic way (Fig. 1a). At the onset of sex determination (Embryonic day or E11.5),  
81 *Runx1* expression was similar in both fetal testis and ovary before becoming ovary-  
82 specific after E12.5 (Fig. 1b), consistent with observations by others<sup>23</sup>. An ovary-  
83 enriched expression of *Runx1* during the window of early gonad differentiation was also  
84 observed in other mammals such as human and goat, as well as in species belonging to  
85 other classes of vertebrates such as the red-eared slider turtle and rainbow trout (Fig.  
86 1c-f), implying an evolutionarily conserved role of RUNX1 in ovary differentiation.

87 To identify the cell types that express *Runx1* in the gonads, we examined a  
88 reporter mouse model that produces EGFP under the control of *Runx1* promoter<sup>27</sup> (Fig.  
89 2). Consistent with the time-course of *Runx1* mRNA expression (Fig. 1b), *Runx1*-EGFP  
90 was present in both fetal ovary and testis at E11.5, and then increased in the ovary and  
91 diminished in the testis at E12.5 onwards (Fig. 2). At E11.5 in both testis and ovary,  
92 *Runx1*-EGFP was present in a subset of SF1+/PECAM- somatic cell population  
93 whereas absent in the SF1-/PECAM+ germ cells (Fig. 2a-d). In the testis, these *Runx1*-  
94 EGFP+ somatic cells corresponded to Sertoli cells, as demonstrated by a complete

95 overlap with SRY, the sex-determining factor that drives Sertoli cell differentiation<sup>28</sup> (Fig.  
96 2e and S1). At this stage, there is no marker for ovarian supporting cells that allow us to  
97 determine which subset of somatic cells were positive for *Runx1*-EGFP in the ovary.  
98 However, at E12.5, when the sex of gonads becomes morphologically distinguishable,  
99 *Runx1*-EGFP was specifically detected in the supporting cell lineage of both sexes:  
100 strongly in FOXL2+ pre-granulosa cells of the ovary (Fig. 2g), and weakly in SOX9+  
101 Sertoli cells of the testis (Fig. 2f and S1). *Runx1*-EGFP expression was eventually  
102 turned off in the fetal testis while maintained in the ovary (Fig. 2h & i). Throughout fetal  
103 development of the ovary, *Runx1*-EGFP remained in FOXL2+ pre-granulosa cells (Fig.  
104 3). *Runx1*-EGFP was also detected in the ovarian surface epithelium at E16.5 and birth  
105 (arrows in Fig. 3b-c), which gives rise to granulosa cells in the cortex of the ovary<sup>29, 30</sup>.  
106 *Runx1*-EGFP was also expressed in somatic cells of the cortical region right underneath  
107 the surface epithelium, and some of these *Runx1*-EGFP+ cells presented a weak  
108 expression of FOXL2 (Fig. 3g-i, arrowheads). In summary, *Runx1* marks the supporting  
109 cell lineage in the gonads at the onset of sex determination, and becomes granulosa  
110 cell-specific as gonads differentiate.

111

## 112 **Loss of *Runx1* leads to ovarian transcriptomic changes resembling those of** 113 ***Foxl2* knockout ovary**

114 The pre-granulosa cell-specific pattern suggests that RUNX1, a factor involved in  
115 cell lineage determination<sup>25</sup>, could contribute to granulosa cell differentiation and  
116 ovarian development. To investigate its specific role in gonadal somatic cells and avoid  
117 early embryonic lethality as a result of global deletion of *Runx1*<sup>31, 32</sup>, we generated a

118 conditional knockout mouse model, in which *Runx1* was ablated in the SF1+ gonadal  
119 somatic cells<sup>33</sup> (Fig. 4). While *Runx1* expression was ablated successfully in the fetal  
120 ovary (Fig. 4a), ovarian morphogenesis appeared normal at birth (Fig. 4b). The  
121 knockout (KO) ovary maintained its typical shape, with FOXL2+ pre-granulosa cells  
122 scattered throughout the ovary and TRA98+ germ cells located mostly in the cortex (Fig.  
123 4b). Despite its normal morphology, *Runx1* KO newborn ovaries exhibited aberrant  
124 transcriptomic profile: expression of 317 genes was altered significantly compared to  
125 the control (Fig. 4c; Dataset S1). The transcriptomic changes of *Runx1* KO ovary were  
126 reminiscent of the ovary lacking *Foxl2*, a conserved gene involved in ovarian  
127 differentiation/maintenance in vertebrates. In the mouse, loss of *Foxl2* results in normal  
128 ovarian morphogenesis at birth despite aberrant ovarian transcriptome<sup>5</sup>. When  
129 comparing the genes changed significantly in the *Runx1* KO (317 genes) with those  
130 affected by the loss of *Foxl2* (749 genes) in newborn ovary, we found that 41% of the  
131 genes differentially expressed in *Runx1* KO (129/317) were also misregulated in the  
132 absence of *Foxl2* (Fig. 4c). The large majority of these 129 genes (93%; 120 genes)  
133 were similarly changed by the loss of either *Runx1* or *Foxl2*: 69% were downregulated  
134 in both KOs (89 genes) and 24% were upregulated in both KOs (31 genes; Dataset S1).  
135 One possible explanation for these common transcriptomic changes in *Runx1* and *Foxl2*  
136 KOs is that *Runx1* could be part of the same signaling cascade as *Foxl2*. However,  
137 analysis of *Runx1* expression in *Foxl2* KO newborn ovaries did not detect any changes  
138 in *Runx1* expression in the absence of *Foxl2* (Fig. 4d). Conversely, *Foxl2* expression  
139 was not changed in the absence of *Runx1*, indicating that *Runx1* and *Foxl2* are  
140 regulated independently of each other in the fetal ovary.



141

142 **Inactivation of both *Runx1* and *Foxl2* results in masculinization of the fetal**  
143 **ovaries**

144 The common transcriptomic changes identified in *Runx1* and *Foxl2* KO ovaries  
145 raised the question whether RUNX1 and FOXL2 could play redundant or  
146 complementary roles in supporting cell differentiation. We therefore generated  
147 *Runx1/Foxl2* double KO mice (hereafter referred as DKO) and compared ovarian  
148 differentiation in the absence of *Runx1*, *Foxl2*, or both (Fig. 5 and S2-3). At E15.5,  
149 differentiation of supporting cells into Sertoli cells in the testis or pre-granulosa cells in  
150 the ovary has already been established. For instance, the transcription factor DMRT1,  
151 which is involved in the maintenance of Sertoli cell identity<sup>18</sup>, is expressed in Sertoli  
152 cells but not pre-granulosa cells (Fig. 5a & e). At this stage, DMRT1 is also present in a  
153 few germ cells in both testis and ovary<sup>34</sup>. Similar to the control ovaries, ovaries lacking  
154 either *Runx1* or *Foxl2* had no DMRT1 proteins in the supporting cells (Fig. 5a-c).  
155 However, the combined loss of *Runx1* and *Foxl2* resulted in aberrant expression of  
156 DMRT1 in the supporting cells of the fetal ovary (Fig. 5d). At birth, *Runx1/Foxl2* DKO  
157 ovary formed structures similar to fetal testis cords in the center, with DMRT1+ cells  
158 surrounding clusters of germ cells (Fig. 5i). Such structure was not observed in *Runx1*  
159 or *Foxl2* single KO ovaries with the exception that DMRT1 protein started to appear in a  
160 few supporting cells in the newborn *Foxl2* KO ovaries, in what appears to be one of the  
161 first signs of postnatal masculinization of *Foxl2* KO ovaries at the protein level (Fig. 5h).  
162 Contrary to DMRT1, SOX9 protein, a key driver of Sertoli cell differentiation<sup>35, 36</sup>, was  
163 not detected in the *Runx1/Foxl2* DKO newborn ovaries (Fig. 6). Our results demonstrate

164 that a combined loss of *Runx1* and *Foxl2* induces partial masculinization of the  
165 supporting cells during fetal development of the ovary.

166 To further characterize the impacts of the combined loss of *Runx1* and *Foxl2* on  
167 ovarian differentiation, we compared the transcriptome of *Runx1/Foxl2* DKO newborn  
168 ovaries with the transcriptomes of control, *Runx1*, or *Foxl2* single KO ovaries (Fig. 7 and  
169 Dataset S2). Between *Runx1/Foxl2* DKO and control ovaries, 918 genes were  
170 differentially expressed, with 499 genes downregulated and 419 genes upregulated in  
171 the DKO ovary (fold-change >1.5;  $p < 0.05$ ; Dataset S3). The heat-map for the 918  
172 differentially expressed genes in *Runx1/Foxl2* DKO ovaries demonstrated allele-specific  
173 impacts with a mild and often non-significant effect in the absence of *Runx1*, an  
174 intermediate/strong effect in the absence of *Foxl2*, and a strongest effect in the absence  
175 of both *Runx1* and *Foxl2* (Fig. 7a). Gene ontology (GO) analysis revealed that the  
176 downregulated genes were associated with “ovarian follicle development” and “female  
177 gonad development” whereas “male sex determination” was the most significantly  
178 enriched process for the upregulated genes (Fig. S4). To determine the contribution of  
179 *Runx1* and *Foxl2* to the transcriptomic changes, the genes significantly downregulated  
180 (Fig. 7b, d & e; Dataset S4) or upregulated (Fig. 7c, f, & g; Dataset S4) were compared  
181 among *Runx1/Foxl2* single and double knockouts. Conforming to the hierarchical  
182 clustering (Fig. 7a), *Foxl2* was the main contributor to the transcriptional changes  
183 observed in *Runx1/Foxl2* DKO: 61% of the genes downregulated in DKO were also  
184 downregulated in *Foxl2* KO (304/499 genes in the overlapping region between purple  
185 and red circle in Fig. 7b) and 43% of the genes upregulated in DKO were also  
186 upregulated in *Foxl2* KO (182/419 genes in the overlapping region between purple and

187 red circle in Fig. 7c). In addition, some genes appeared to be controlled by both *Foxl2*  
188 and *Runx1*, and were significantly downregulated (66 genes in the overlapping region  
189 between the three circles in Fig. 7b) or upregulated (29 genes in the overlapping region  
190 between the three circles in Fig. 7c) in all three knockouts. For instance, the genes *Fst*  
191 and *Cyp19a*, both involved in granulosa cell differentiation/function<sup>37, 38</sup>, were  
192 downregulated in *Runx1* KO, *Foxl2* KO, and more repressed in *Runx1/Foxl2* DKO (Fig.  
193 7d). On the other hand, desert hedgehog (*Dhh*) was upregulated in all 3 knockouts, with  
194 highest expression in the DKO (Fig. 7f). Notably, 30% of the genes downregulated  
195 (151/499 genes; Fig. 7b) and 52% of the genes upregulated (219/419 genes; Fig. 7c) in  
196 *Runx1/Foxl2* DKO were not significantly changed in *Runx1* or *Foxl2* single KO. Most of  
197 the genes in this category were mildly changed in the single knockouts without reaching  
198 the cut-off of the microarray analysis. For instance, *Foxp1*, a gene whose expression is  
199 enriched in pre-granulosa cells<sup>39</sup>, was significantly downregulated only in *Runx1/Foxl2*  
200 DKO (Fig. 7e) whereas *Fgf9*, a Sertoli gene contributing to testis differentiation<sup>40</sup> and  
201 *Pdgfc* were significantly upregulated only in *Runx1/Foxl2* DKO (Fig. 7g).

202 Comparing to *Foxl2* single KO female (Fig. 5), in which sex reversal only became  
203 apparent postnatally<sup>5</sup>, *Runx1/Foxl2* DKO female exhibited masculinization of the ovaries  
204 with visible morphological changes before birth. To determine how the loss of *Runx1*  
205 contributed to the early masculinization of *Runx1/Foxl2* DKO ovaries, we identified the  
206 potential RUNX1-regulated genes by comparing the transcriptomes of *Runx1/Foxl2*  
207 DKO and *Foxl2* single KO ovaries. A total of 218 genes were differentially expressed  
208 between *Runx1/Foxl2* DKO and *Foxl2* KO ovaries, with 114 genes significantly  
209 upregulated, and 104 genes significantly downregulated in the DKO (fold-change >1.5;

210 p<0.05; Fig. 7h and Dataset S5). Expression of most of these genes was already  
211 altered in *Foxl2* single KO ovaries; however, the additional loss of *Runx1* exacerbated  
212 their mis-expression. For instance, the pro-testis gene *Dmrt1* and *Nr5a1* were  
213 significantly upregulated whereas the granulosa-cell enriched transcripts *Fst* and *Ryr2*  
214 were further downregulated at birth (Fig. 7d & i). On the other hand, the additional loss  
215 of *Runx1* did not cause further upregulation of the Sertoli genes *Sox9* and *Amh* at birth,  
216 suggesting that *Runx1* does not contribute to their repression in the ovary (Fig. 7j).  
217 Overall, the transcriptomic analyses of *Runx1/Foxl2* single and double knockouts reveal  
218 that the additional loss of *Runx1* amplifies the mis-expression of genes already altered  
219 by the sole loss of *Foxl2*.

220

### 221 **RUNX1 shares genome-wide chromatin occupancy with FOXL2 in the fetal ovary**

222 The masculinization of *Runx1/Foxl2* DKO fetal ovaries and the transcriptomic  
223 comparisons of *Runx1/Foxl2* single and double KO ovaries suggest some interplay  
224 between RUNX1 and FOXL2 to control granulosa cell identity. The fact that RUNX1 and  
225 FOXL2 are both transcription factors raised the question whether this interplay could  
226 occur at the chromatin level. We have previously identified the chromatin occupancy of  
227 FOXL2 during ovarian differentiation by chromatin immunoprecipitation followed by  
228 whole genome sequencing or ChIP-seq<sup>41</sup>. We performed additional *de novo* motif  
229 analyses on the genomic regions bound by FOXL2 in the fetal ovary, and discovered  
230 that several other DNA motifs were co-enriched with the FOXL2 DNA motif (Fig. 8a).  
231 Among them, RUNX DNA motif was the second most significantly co-enriched motif.  
232 The other motifs were for CTCF, a factor involved in transcriptional regulation, enhancer

233 insulation and chromatin architecture<sup>42</sup>, and for the DNA motif recognized by multiple  
234 members of the nuclear receptor family including liver receptor homolog-1 (LRH1  
235 encoded by *Nr5a2*) and SF1 (encoded by *Nr5a1*), a known co-factor of FOXL2<sup>43, 44</sup>.  
236 DNA motifs for TEAD transcription factors of the Hippo pathway, ETS, NFYA and  
237 GATA4 were also significantly enriched. The enrichment of RUNX motif with FOXL2  
238 binding motif suggests that RUNX1, the only RUNX enriched in pre-granulosa cells,  
239 could bind similar genomic regions to FOXL2 in the fetal ovary. To confirm this  
240 hypothesis, we performed ChIP-seq for RUNX1 in E14.5 ovaries (Dataset S6), the  
241 same stage the FOXL2 ChIP-seq data were obtained<sup>41</sup>. The top *de novo* motif identified  
242 in RUNX1 ChIP-seq ( $p < 1e-559$ ) matched the RUNX motif<sup>45</sup> (Fig. 8b), and corresponded  
243 to the motif that was co-enriched with FOXL2 in FOXL2 ChIP-seq (Fig. 8a). A total of  
244 10,494 RUNX1 binding peaks were identified in the fetal ovary, with the majority of the  
245 peaks located either in the gene body (Fig. 8c; 25% exon and 22% intron), or close  
246 upstream of the transcription start site TSS (30% Promoter: <1kb of TSS; 12%  
247 Upstream: -10 to -1kb of TSS). Comparison of genome-wide chromatin binding of  
248 RUNX1 and FOXL2 in the fetal ovary revealed significant overlap: 54% (5,619/10,494)  
249 of RUNX1 peaks overlapped with FOXL2 peaks (Fig. 8d).

250       The transcriptomic data from *Runx1/Foxl2* DKO ovaries provided us a list of  
251 genes significantly changed as a result of the absence of *Runx1*, *Foxl2*, or both (Fig. 7).  
252 To identify potential direct target genes of RUNX1 or/and FOXL2, we focused on the  
253 918 genes differentially expressed in *Runx1/Foxl2* DKO ovaries, and determined which  
254 genes were nearest to RUNX1 or/and FOXL2 binding peaks (Fig. 9a and Dataset S7).  
255 More than 50% of these genes (492/918; Fig. 9a) were the closest gene to RUNX1

256 or/and FOXL2 peak. Some of these genes were nearest to only FOXL2 peaks (116  
257 genes in Fig. 9a). For example, *Pla2r1*, a transcript enriched in pre-granulosa cells<sup>39</sup>  
258 and similarly downregulated in both *Foxl2* KO and *Runx1/Foxl2* DKO fetal ovaries (Fig.  
259 9b), contained two FOXL2-specific peaks, one in the promoter and one in the first  
260 intron. On the other hand, 102 genes (Fig. 9a) had RUNX1 specific peaks near their  
261 genomic locations. For instance, *Ryr2*, another transcript enriched in pre-granulosa  
262 cells<sup>39</sup>, was strongly downregulated in both *Runx1* KO and *Runx1/Foxl2* DKO fetal and  
263 newborn ovaries (Fig. 7i and Fig. 9c), and contained one RUNX1 specific peak in its  
264 intronic region. Finally, 274 genes were the closest genes to peaks for both RUNX1 and  
265 FOXL2, with the majority of them (197 genes) nearest to overlapping peaks for RUNX1  
266 and FOXL2 (Fig. 9a). Most of these genes (74%; 146/197 genes) were downregulated  
267 in *Runx1/Foxl2* DKO ovaries (Dataset S7). For instance, the granulosa cell enriched  
268 genes *Fst* and *Itpr2*, both downregulated in *Runx1/Foxl2* single and double KO ovaries  
269 (Fig. 7d and 9d), contained common binding peaks for FOXL2 and RUNX1 (Fig. 9a and  
270 9d). For *Fst*, this binding of RUNX1 and FOXL2 was located in its first intron, in the  
271 previously identified regulatory region that contributes to its expression<sup>41, 46</sup>. On the  
272 other hand, the Sertoli cell gene *Dmrt1*, strongly upregulated in *Runx1/Foxl2* DKO (Fig.  
273 5 and 7i), contained a common binding site for FOXL2 and RUNX1 near its promoter  
274 (Fig. 9a). Taken together, our results reveal that RUNX1, a transcription factor  
275 expressed in the fetal ovary of various vertebrate species, contributes to ovarian  
276 differentiation and maintenance of pre-granulosa cell identity through an interplay with  
277 FOXL2 that occurs at the chromatin level.  
278

## 279 Discussion

### 280 RUNX1 is a part of the multi-component network that controls pre-granulosa cell 281 identity

282 The molecular events that specify granulosa cell fate are complex and non-linear,  
283 involving several signaling pathways that have redundant and complementary functions.  
284 This is in contrast to the fetal testis, where the molecular pathway driving its  
285 differentiation appears linear and sequential. Removal of one of the top pieces in the  
286 testis differentiation pathway has a domino effect that prevents induction of downstream  
287 events. This is exemplified by the complete gonadal sex-reversal in gain- or loss-of-  
288 function mouse models for SRY or SOX9, the two transcription factors responsible for  
289 the initiation of the testis morphogenesis<sup>28, 35, 47</sup>. This is not the case in the mouse ovary,  
290 where no single-gene loss/mutation results in a complete ovary-to-testis sex-reversal.  
291 For instance, defects in the WNT4/RSP01/beta-catenin or loss of *Foxl2* causes a late or  
292 postnatal partial ovary-to-testis sex-reversal, while the combined loss of *Foxl2* and  
293 elements of the WNT4/RSP01/beta-catenin pathway (*Wnt4* or *Rspo1*) leads to ovary-  
294 to-testis sex-reversal more pronounced than each single knockout model in the mouse<sup>8</sup>,  
295 <sup>9</sup>. In this study, we demonstrated that *Runx1* contributes to the molecular network  
296 controlling pre-granulosa cell differentiation. Loss of *Runx1* in somatic cells of the  
297 ovaries altered ovarian transcriptome but did not affect ovarian morphogenesis at birth.  
298 In contrast, the combined loss of *Runx1* and *Foxl2* compromised pre-granulosa cells  
299 identity. Loss of *Runx1* or *Foxl2* affected a common set of genes, and these  
300 transcriptomic changes were enhanced in the absence of both genes, reaching a  
301 threshold that masculinized the fetal ovary. One of the most striking changes was the

302 expression of DMRT1 in the fetal supporting cells. DMRT1 is a key driver of Sertoli cell  
303 differentiation and testis development in various species<sup>15, 20</sup>. In the fly, *doublesex (dsx)*,  
304 an ortholog of mammalian *DMRT1*, controls testis differentiation<sup>15</sup>. Intriguingly, *runt*, the  
305 fly ortholog of *RUNX1*, drives ovarian differentiation by antagonizing the testis-specific  
306 transcriptional regulation of *dsx*<sup>21</sup>. In the mouse, testis differentiation is not controlled by  
307 DMRT1 but by SOX transcription factors SRY and its direct target SOX9. However,  
308 RUNX1 does not appear to contribute to the repression of the key pro-testis gene *Sox9*  
309 in the fetal ovary and SOX9 protein was not detected in *Runx1/Foxl2* DKO ovary at  
310 birth. This is in contrast with the phenotype of *Wnt4/Foxl2* DKO newborn ovaries where  
311 SOX9 was strongly upregulated, and as a consequence the ovaries were more  
312 masculinized<sup>9</sup>. Overall, our findings suggest that slightly different pro-ovarian networks  
313 control the repression of the evolutionary conserved pro-testis genes *Sox9* and *Dmrt1*:  
314 *Sox9*, which plays a primary role in Sertoli cell differentiation in the mouse, is repressed  
315 by an interplay between the WNT4/RSP01/beta-catenin and FOXL2<sup>8, 9</sup>. On the other  
316 hand, *Dmrt1*, which has taken a secondary role in Sertoli cell differentiation in the  
317 mouse, is repressed by an interplay between RUNX1 and FOXL2.

318 Seeking the mechanisms underlying the interplay between RUNX1 and FOXL2,  
319 we identified that RUNX DNA binding motif is significantly co-enriched with FOXL2 motif  
320 in genomic regions bound by FOXL2 in the fetal ovary. Moreover, RUNX1 genome-wide  
321 occupancy partially overlaps with FOXL2 in the fetal ovary, suggesting that they bind  
322 common regulatory regions to control granulosa-cell identity and ovarian development.  
323 By themselves, RUNX proteins are weak transcription factors, and they require other  
324 transcriptional regulators to function as either repressors or activators of transcription<sup>26</sup>.



325 Interplay between RUNX1 and several members of the forkhead transcription factor  
326 family has been documented in different tissues. For instance, RUNX1 is a co-activator  
327 of FOXO3 in hepatic cells<sup>48</sup>. Similarly, an interplay between RUNX1 and  
328 FOXO1/FOXO3 was demonstrated in breast epithelial cells where a subset of FOXO  
329 target genes were jointly regulated with RUNX1<sup>49</sup>. Another forkhead protein, FOXP3,  
330 acts with RUNX1 to control gene expression in T-cells<sup>50</sup> and breast epithelial cells<sup>51</sup>.  
331 Such cooperation in various tissues suggest that the interplay between RUNXs and  
332 forkhead transcription factors maybe an evolutionary conserved phenomenon. In  
333 addition to the genes co-regulated with FOXL2, we identified genes that were  
334 specifically mis-expressed in the absence of *Runx1* but not *Foxl2*. Genome-wide  
335 analyses of RUNX1 binding in the fetal ovary also identified genomic regions bound by  
336 RUNX1 but not FOXL2. These results suggest that RUNX1 could also contribute to  
337 ovarian development or function independently of FOXL2.

338

### 339 **RUNX1, a marker of gonadal supporting cell identity**

340 RUNX1 contributes to cell fate determination in various developmental processes  
341 such as hematopoiesis and hair follicle development. Depending on its interplay with  
342 other signal transduction pathways or co-factors, RUNX1 controls which path the  
343 precursor cells take when they are at the crossroad between cell proliferation/renewal  
344 and lineage-specific commitment<sup>25</sup>. We discovered that *Runx1* has an ovary-biased  
345 expression during gonad differentiation in various vertebrate species, including turtle,  
346 rainbow trout, goat, mouse, and human. In the mouse embryonic gonads, *Runx1* is first  
347 detected in the supporting cells in a non-sexually dimorphic way at the onset of sex

348 determination. While its expression is maintained in the ovary, *Runx1* appears to be  
349 actively repressed in the testis between E11.5 and E12.5 as the supporting cells commit  
350 to Sertoli cell fate. The suppression of *Runx1* in the fetal testis is corroborated by  
351 previously published data from a time-course transcriptomic analysis during early gonad  
352 development<sup>52</sup> and single-cell sequencing analysis of SF1+ progenitor cells<sup>53</sup>. The time-  
353 course of Sertoli cell differentiation at the single-cell level revealed that *Runx1* follows  
354 an identical spatiotemporal pattern of expression with *Sry*<sup>53</sup>. In the mouse, *Sry*  
355 expression in Sertoli cells is quickly turned off after the initiation of testis differentiation,  
356 and it is suspected that the repression of *Sry* is due to a negative feedback loop by  
357 downstream pro-testis genes. The similar pattern of downregulation of *Runx1* in the  
358 testis after E11.5 raises the possibility that *Runx1* is downregulated by a similar  
359 signaling pathway. Regulation of *Runx1* gene expression is complex, and several  
360 enhancers that confer tissue-specific expression have been identified<sup>54</sup>. It remains to be  
361 determined how *Runx1* expression is controlled in the gonads, and how it is actively  
362 repressed in the fetal testis.

363 In contrary to the testis, the fetal ovary maintains expression of *Runx1* in the  
364 supporting cells as they differentiate into granulosa cells. During ovarian differentiation,  
365 granulosa cells arise from two different waves at different stages of development: the  
366 first cohort of granulosa cells arises from the bipotential supporting cell precursors that  
367 are able to differentiate into either Sertoli cells or pre-granulosa cells during sex  
368 determination<sup>55</sup>. The second wave of granulosa cells that eventually populate the  
369 cortical region of the ovary appears later in gestation. These cells of the second wave  
370 arise from LGR5+ cells of the ovarian surface epithelium that ingress into the ovary from

371 E15.5 to postnatal day 4 and eventually become LGR5-/FOXL2+ granulosa cells<sup>29, 30,</sup>  
372 <sup>56</sup>. This timing of the establishment of the second cohort of granulosa cells correlates  
373 with the expression of *Runx1*-EGFP in a subset of cells in the surface epithelium and in  
374 granulosa cells of the ovarian cortex at E16.5 and birth. These results suggest that  
375 *Runx1* marks granulosa cell precursors that will give rise to the second wave of FOXL2+  
376 granulosa cells in the cortex. Therefore, both expression at onset of sex determination  
377 and at the surface epithelium/cortex at the time of the second wave of granulosa cells  
378 recruitment suggest that *Runx1* is activated in cells that are primed to become  
379 supporting/granulosa cells.

380

### 381 **Towards the identification of granulosa cell genomic signatures**

382 Multiple transcription factors, rather than a single one, often form complex  
383 genetic regulatory networks that control cell fate determination. Genomic sequence  
384 motifs or cis-regulatory elements for Sertoli cells, the supporting cell lineage in the  
385 testis, were identified by combined analyses of SOX9 and DMRT1 ChIP-seq and by  
386 motif prediction<sup>57</sup>. These “Sertoli cell signatures” are composed of organized binding  
387 motifs for transcription factors critical for Sertoli cell differentiation, including SOX9,  
388 GATA4 and DMRT1. These Sertoli cell signatures, present in mammals and other  
389 vertebrates, could represent a conserved regulatory code that governs the cascade of  
390 Sertoli cell differentiation, regardless whether it primarily relies on SOX transcription  
391 factors like SRY in mammals, or on DMRT1 like in several vertebrate species. Similarly,  
392 one would expect the presence of conserved “granulosa cell signature” genomic regions  
393 that confers granulosa cell differentiation. Since FOXL2 is a highly conserved gene in

394 granulosa cell differentiation in vertebrates, we used FOXL2 as an anchor factor to  
395 identify other factors that could take part in the regulatory network controlling granulosa  
396 cell differentiation/function. Unbiased analyses of the motifs co-enriched with FOXL2  
397 motif in the fetal ovary identified the RUNX motif as one of the most co-enriched motifs.  
398 In addition to the RUNX motif, motifs for CTCF, nuclear receptors SF-1/LRH-1/ESRRB  
399 and transcription factors TEADs and GATAs were also significantly enriched with  
400 FOXL2 consensus motif in FOXL2-bound chromatin regions. For many of these  
401 transcription factors, their potential role in gonad differentiation is unknown or limited.  
402 For example, the transcription factors of the TEAD family belong to the Hippo pathway,  
403 which is involved in the regulation of Sertoli cell gene expression in the fetal gonads<sup>58</sup>.  
404 However, the potential involvement of the hippo pathway in granulosa cell differentiation  
405 has not been investigated.

406 In conclusion, we identified RUNX1 as a transcription factor involved in pre-  
407 granulosa cell differentiation. RUNX1 delineates the supporting cell lineage and  
408 contributes to granulosa cell differentiation and maintenance of their identity through an  
409 interplay with FOXL2. Our findings provide new insights into the genomic control of  
410 granulosa cell differentiation, and pave the way for the identification of novel  
411 transcription factors and cis-signatures contributing to the fate determination of  
412 granulosa cells and the consequent formation of a functional ovary.

## 413 **Methods**

### 414 **Mouse models**

415 Tg(*Runx1*-EGFP) reporter mouse was purchased from MMRRC (MMRRC\_010771-  
416 UCD), and CD-1 mice were purchased from Charles River (stock number 022). *Runx1*<sup>+/-</sup>  
417 (B6.129S-*Runx1*<sup>tm1Spe/J</sup>) and *Runx1*<sup>ff</sup> (B6.129P2-*Runx1*<sup>tm1Tani/J</sup>) mice were purchased  
418 from the Jackson Laboratory (stock numbers 005669 and 008772, respectively). *Sf1*-  
419 Cre<sup>Tg/Tg</sup> mice<sup>33</sup> were provided by late Dr. Keith Parker and *Foxl2*<sup>+/-</sup> mice<sup>59</sup> by Dr. David  
420 Schlessinger (National Institute on Aging), respectively. *Runx1* KO mice (*Sf1Cre*<sup>+Tg</sup>;  
421 *Runx1*<sup>f/-</sup>) were generated by crossing *Runx1*<sup>ff</sup> females with *Sf1-Cre*<sup>+Tg</sup>; *Runx1*<sup>+/-</sup> males.  
422 Controls were *Sf1-Cre*<sup>+/+</sup>; *Runx1*<sup>+f</sup> littermates. *Runx1*/*Foxl2* double knockout mice  
423 (*Sf1Cre*<sup>+Tg</sup>; *Runx1*<sup>f/-</sup>; *Foxl2*<sup>-/-</sup>) were generated by crossing *Runx1*<sup>ff</sup>; *Foxl2*<sup>+/-</sup> females with  
424 *Sf1Cre*<sup>+Tg</sup>; *Runx1*<sup>+/-</sup>; *Foxl2*<sup>+/-</sup> males. This cross also generated the single knockouts for  
425 *Runx1* (*Sf1Cre*<sup>+Tg</sup>; *Runx1*<sup>f/-</sup>; *Foxl2*<sup>+/+</sup>) and *Foxl2* (*Sf1Cre*<sup>+/+</sup>; *Runx1*<sup>f/+</sup>; *Foxl2*<sup>-/-</sup>), and  
426 control littermates (*Sf1-Cre*<sup>+/+</sup>; *Runx1*<sup>+f</sup>; *Foxl2*<sup>+/+</sup>). Time-mating was set up by housing  
427 female mice with male mice overnight and the females were checked for the presence  
428 of vaginal plug the next morning. The day when the vaginal plug was detected was  
429 considered embryonic day E0.5. All experiments were performed on at least four  
430 animals for each genotype. All animal procedures were approved by the National  
431 Institutes of Health Animals Care and Use Committee, and were performed in  
432 accordance with an approved National Institute of Environmental Health Sciences  
433 animal study proposal.

434

### 435 **Immunofluorescences**

436 For the Tg(*Runx1*-EGFP) mice, gonads were collected and fixed in 4%  
437 paraformaldehyde for 1-2h at room temperature. Immunofluorescence experiments  
438 were performed on whole gonads at E11.5 and E12.5 and on 8  $\mu$ m cryosections for  
439 E14.5, E16.5 and P0 (birth) gonads. The EGFP was detected in wholemount gonads by  
440 direct fluorescent imaging, and an anti-GFP antibody was used for  
441 immunofluorescences on sections. For the different knockout models, gonads were  
442 fixed in 4% paraformaldehyde overnight at 4°C. Immunofluorescence experiments were  
443 performed on 7  $\mu$ m paraffin sections of E15.5 and P0 gonads as previously described<sup>60</sup>.  
444 The antibodies used in this study are listed in Table S1. Whole gonads and sections  
445 were imaged under a Leica DMI4000 confocal microscope.

446

#### 447 **Real-Time PCR analysis in the mouse**

448 For the time-course kinetics of *Runx1* expression, fetal gonads from CD-1 embryos at  
449 embryonic day E11.5, E12.5, E13.5, E14.5, E16.5, E18.5 and postnatal day P3 were  
450 separated from the mesonephros and snap-frozen. For each stage, 3 biological  
451 replicates were collected, with 6 gonads/replicate for the E11.5 stage and 3  
452 gonads/replicate for the other stages. For *Runx1* KO analysis, control and KO ovaries  
453 were collected at E14.5 (n = 4 biological replicates/genotype). For *Runx1/Foxl2* DKO  
454 analysis, control, *Runx1* and *Foxl2* single and double KO ovaries were collected at  
455 E15.5 (n=4/genotype) and P0 (n=5/genotype). For all experiments, total RNA was  
456 isolated for each replicate using PicoPure RNA isolation kit (Arcturus, Mountain View,  
457 CA), RNA quality and concentration were determined using the Nanodrop 2000c and  
458 300 to 400 ng of RNA was used for cDNA synthesis with the Superscript II cDNA

459 synthesis system (Invitrogen Corp., Carlsbad, CA). Gene expression was analyzed by  
460 real-time PCR using Bio-Rad CFX96™ Real-Time PCR Detection system. Gene  
461 expression was normalized to *Gapdh*. The Taqman probes and primers used to detect  
462 transcript expression are listed in Table S1. Data were analyzed using Prism GraphPad  
463 Software by unpaired Student's *t*-test or by ANOVA  $p < 0.05$ . Values are presented as  
464 mean  $\pm$  s.e.m.

465

### 466 ***Runx1* expression in other species**

467 For the rainbow trout, *Runx1* expression during gonadal development was assessed by  
468 quantitative PCR, as previously described<sup>61</sup>. Species-specific primers used are listed in  
469 Table S1. For the red-eared slider turtle, *Runx1* expression during gonadal development  
470 was assessed at Female-Promoting Temperature (FPT) of 31°C and at Male-Promoting  
471 Temperature (MPT) of 26°C by RNA-seq, as previously described<sup>62</sup>. For the goat,  
472 *Runx1* expression during gonadal development was assessed by quantitative PCR, and  
473 2 to 3 biological replicates were used for each stage of development as previously  
474 described<sup>63</sup>. Values are presented as mean  $\pm$  s.d. All goat handling procedures were  
475 conducted in compliance with the guidelines on the Care and Use of Agricultural  
476 Animals in Agricultural Research and Teaching in France (Authorization no. 91-649 for  
477 the Principal Investigator, and national authorizations for all investigators. Approval from  
478 the Ethics Committee: 12/045). For the human, *Runx1* expression during gonadal  
479 development was assessed by RNA-seq (Lecluze *et al.* in preparation). Human fetuses  
480 (6-12 GW) were obtained from legally-induced normally-progressing terminations of  
481 pregnancy performed in Rennes University Hospital in France. Tissues were collected

482 with women's written consent, in accordance with the legal procedure agreed by the  
483 National agency for biomedical research (authorization #PFS09-011; Agence de la  
484 Biomédecine) and the approval of the Local ethics committee of Rennes Hospital in  
485 France (advice # 11-48).

486

#### 487 **Microarray analysis**

488 Gene expression analysis of control, *Runx1* KO, *Foxl2* KO and *Runx1/Foxl2* DKO  
489 ovaries was conducted using Affymetrix Mouse Genome 430 2.0 GeneChip® arrays  
490 (Affymetrix, Santa Clara, CA) on 4 biological replicates (one P0 gonad per replicate) for  
491 each genotype. Fifty (50) nanograms of total RNA were amplified and labeled as  
492 directed in the WT-Ovation Pico RNA Amplification System and Encore Biotin Module  
493 protocols. Amplified biotin-aRNA (4.6 µg) was fragmented and hybridized to each array  
494 for 18 hours at 45°C in a rotating hybridization. Array slides were stained with  
495 streptavidin/phycoerythrin utilizing a double-antibody staining procedure and then  
496 washed for antibody amplification according to the GeneChip Hybridization, Wash and  
497 Stain Kit and user manual following protocol FS450-0004. Arrays were scanned in an  
498 Affymetrix Scanner 3000 and data was obtained using the GeneChip® Command  
499 Console Software (AGCC; Version 3.2) and Expression Console (Version 1.2).  
500 Microarray data have been deposited in GEO under accession code GSE129038. Gene  
501 expression analyses were conducted with Partek software (St. Louis, Missouri) using a  
502 one-way ANOVA comparing the RMA normalized log<sub>2</sub> intensities. A full dataset Excel  
503 file containing the normalized log<sub>2</sub> intensity of all genes for each genotype, and a  
504 graphic view of their expression is provided in Dataset S2. In order to identify



505 differentially expressed genes, analysis of variance (ANOVA) was used to determine if  
506 there was a statistical difference between the means of groups and the gene lists were  
507 filtered with  $p < 0.05$  and fold-change cutoff of 1.5. The heat-map was created comparing  
508 the genes that were significantly different between control and *Runx1/Foxl2* double  
509 knockout ovaries. Venn diagrams were generated in Partek by comparing gene-  
510 symbols between the lists of genes differentially expressed.

511

### 512 **ChIP-seq assays and analysis**

513 Ovaries from E14.5 CD-1 embryos were separated from the mesonephros, snap-frozen,  
514 and stored at  $-80^{\circ}\text{C}$ . RUNX1 ChIP-seq experiments and analyses in E14.5 ovaries were  
515 performed as previously described for FOXL2 ChIP-seq<sup>41</sup>. Two independent ChIP-seq  
516 experiments were performed by Active Motif Inc. using 20-30  $\mu\text{g}$  of sheared chromatin  
517 from pooled embryonic ovaries ( $n=100-120$  ovaries/ChIP), and 10  $\mu\text{l}$  of RUNX1  
518 antibody<sup>64</sup> (provided by Drs. Yoram Groner and Ditsa Levanon, the Weizmann Institute  
519 of Science, Israel). ChIP-seq libraries were sequenced as single-end 75-mers by  
520 Illumina NextSeq 500, then filtered to retain only reads with average base quality score  
521  $>20$ . Reads were mapped against the mouse mm10 reference genome using Bowtie<sup>65</sup>  
522 v1.2 with parameter “-m 1” to collect only uniquely-mapped hits. Duplicate mapped  
523 reads were removed using Picard tools MarkDuplicates.jar (v1.110). The number of  
524 uniquely-mapped non-duplicate reads for each biological replicate was 8,932,674 and  
525 15,036,698. After merging the replicate datasets, binding regions were identified by  
526 peak calling using HOMER v.4.9<sup>66</sup> with  $\text{FDR} < 1\text{e-}5$ . Called peaks were subsequently re-  
527 defined as 300mers centered on the called peak midpoints and filtered for a 4-fold

528 enrichment over input and over local signal. Genomic distribution of RUNX1-bound  
529 regions was determined based on Refseq gene models as downloaded from the UCSC  
530 Genome Browser as of August 09, 2017. Enriched motifs were identified using HOMER  
531 findMotifsGenome.pl de novo motif analysis with parameter “-size given”. For RUNX1  
532 and FOXL2 ChIP-seq comparisons, binding peaks that had at least 1 bp in common  
533 were considered overlapping. Peaks were assigned to the nearest gene based on  
534 RefSeq. Gene lists were analyzed for enrichment using the online tool EnrichR<sup>67</sup>. The  
535 ChIP-seq data are available in the ReproGenomics Viewer (<https://rgv.genouest.org>)<sup>68</sup>,  
536 <sup>69</sup> and Gene Expression Omnibus (GSE128767; <http://www.ncbi.nlm.nih.gov/geo/>).  
537

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### 783 **Authors contributions**

784 B.N. performed the experiments in the mouse; B.N. and H.H.-C.Y. designed the study,  
785 analyzed data and wrote the paper; S.A.G performed bioinformatic analyses; F.C and  
786 E.L. analyzed *RUNX1* expression in human fetal gonads; M.P. and E.P. analyzed  
787 *RUNX1* expression in the goat; E.D-D-P. and Y.G. analyzed *runx1* expression in  
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790

### 791 **Competing interests**

792 The authors declare no competing financial or non-financial interests.

793

### 794 **Materials & Correspondence**

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807

808 **Figure legends**

809

810 **Figure 1: *RUNX1* expression is enriched in female gonads during gonadal**  
811 **differentiation in the mouse and other vertebrates**

812 **(a)** Expression of *Runx1*, *Runx2* and *Runx3* mRNAs in ovaries and testis of E14.5  
813 mouse embryos (n= 5/sex); Values are presented as mean  $\pm$  s.e.m.; non-parametric t-  
814 test,  $**P<0.01$ ; ns: not significant. **(b)** Expression time course of *Runx1* mRNA in mouse  
815 gonads during gonadal differentiation (n=3/stage); Values are presented as mean  $\pm$   
816 s.e.m. **(c-f)** Time course of *RUNX1* mRNA expression in four other vertebrate species,  
817 human, goat, red-eared slider turtle and rainbow trout during gonad differentiation. For  
818 the turtle, pink and blue bars represent gonads at Female-Promoting Temperature FPT  
819 of 31°C and at Male-Promoting Temperature MPT of 26°C respectively<sup>62</sup>. *RUNX1*  
820 expression was analyzed by RNA-seq in human and red-eared slider turtle<sup>62</sup>, and by  
821 qPCR in the goat and rainbow trout. Green highlighted areas represent the window of  
822 early gonadal differentiation.

823

824 **Figure 2: *Runx1* is expressed in the supporting cell lineage during gonad**  
825 **differentiation in the mouse embryos**

826 **(a-g)** Whole mount immunofluorescence of testes and ovaries from Tg(*Runx1*-EGFP)  
827 reporter mice at E11.5 and E12.5. Gonads with endogenous EGFP were co-labeled  
828 with markers for germ cells/vasculature (PECAM-1; a-b), somatic cells (SF1; c-d),  
829 Sertoli cells in the testis (SRY in e and SOX9 in f), and for granulosa cells in the ovary  
830 (FOXL2; g). Scale bars: 100  $\mu$ m. **(h-i)** Detection of endogenous EGFP in freshly  
831 collected E14.5 gonads. Scale bars: 200  $\mu$ m. Dotted lines outline the gonads.

832

833 **Figure 3: *Runx1* expression is maintained in granulosa cells throughout fetal**  
834 **ovarian development**

835 **(a-c)** Immunofluorescence for EGFP on Tg(*Runx1*-EGFP) ovary sections at E14.5,  
836 E16.5 and birth. Scale bars: 50  $\mu$ m. **(d-i)** Immunofluorescence for EGFP and the  
837 granulosa cell marker FOXL2 at E14.5 (d-f) and E16.5 (g-i), corresponding to the white  
838 square outlined areas in (a) and (b) respectively. *Runx1* is expressed in granulosa cells  
839 throughout ovarian development and the surface epithelium after E14.5. Dotted lines  
840 outline the gonads. Arrows: EGFP+ ovarian surface epithelium. Arrowheads:  
841 EGFP/FOXL2 double positive cells. Scale bars: 50  $\mu$ m.

842

843 **Figure 4: *Runx1* KO newborn ovaries present normal morphogenesis but share**  
844 **common transcriptomic changes with *Foxl2* KO ovaries.**

845 **(a)** Validation of *Runx1* knockout in E14.5 fetal ovaries by quantitative PCR (n=5);  
846 unpaired Student's t-test \*\*\* $P < 0.001$ . Values are presented as mean  $\pm$  s.e.m. **(b)**  
847 Immunofluorescence for granulosa cell marker FOXL2, germ cell marker TRA98 and  
848 nuclear counterstain DAPI (blue) in control and *Runx1* KO ovaries at birth (P0). Scale  
849 bar: 100  $\mu$ m. **(c)** Venn diagram comparing the 317 genes differentially expressed in  
850 newborn *Runx1* KO vs. Control ovaries (green circle) with the 749 genes differentially  
851 expressed in newborn *Foxl2* KO vs. Control ovaries (purple circle). Genes differentially  
852 expressed were identified by microarray (n=4/genotype; fold change  $> 1.5$ ,  $p < 0.05$ ). **(d)**  
853 Quantitative PCR analysis of *Runx1* and *Foxl2* mRNA expression in control, *Runx1* KO,

854 and *Foxl2* KO newborn ovaries (n=5/genotype). Values are presented as mean  $\pm$  s.e.m.  
855 One-way ANOVA,  $P < 0.05$ . Bars with different letters (a, b) are significantly different.

856

857 **Figure 5: Combined loss of *Runx1* and *Foxl2* results in masculinization of the**  
858 **fetal ovaries.**

859 **(a-j)** Immunofluorescence for the Sertoli cell and germ cell marker DMRT1, the germ  
860 cell marker TRA98 and the granulosa cell marker FOXL2 in control, *Runx1* KO, *Foxl2*  
861 KO, *Runx1/Foxl2* double knockout ovaries and control testes at E15.5 **(a-e)** and birth **(f-**  
862 **j)**. The grey represents DAPI nuclear staining. Dotted lines outline the gonads. Higher  
863 magnifications are shown for the outlined boxes in **a-e** and **f-j** respectively. Scale bars:  
864 100  $\mu$ m.

865

866 **Figure 6: SOX9 protein is not detected in *Runx1/Foxl2* DKO newborn ovaries**

867 Immunofluorescence for Sertoli cell markers DMRT1 and SOX9 and germ cell marker  
868 TRA98 on consecutive sections in control testis (a & c) and *Runx1/Foxl2* DKO ovary (b  
869 & d) at birth. Scale bar: 50  $\mu$ m.

870

871 **Figure 7: Comparison of *Runx1* KO, *Foxl2* KO and *Runx1/Foxl2* double KO**  
872 **transcriptomes.**

873 **(a)** Heat-map for the 918 genes differentially expressed in *Runx1/Foxl2* double knockout  
874 (DKO) vs. control (Ctr) newborn ovaries. The heat map shows the expression of these  
875 918 genes in control, *Runx1* KO, *Foxl2* KO and *Runx1/Foxl2* DKO ovaries (microarray;  
876 n=4/genotype; One-way ANOVA; fold change  $>1.5$ ,  $P < 0.05$ ). **(b-c)** Venn diagram

877 comparing the genes downregulated (**b**) or upregulated (**c**) in *Runx1* KO (green circle),  
878 *Foxl2* KO (purple circle) and *Runx1/Foxl2* DKO (purple circle) newborn ovaries. (**d-g**)  
879 Validation by quantitative PCR of genes identified in the Venn diagrams as significantly  
880 downregulated in all three KO (**d**), or only in *Runx1/Foxl2* DKO (**e**), or significantly  
881 upregulated in all in all three KO (**f**) or only in *Runx1/Foxl2* DKO (**g**). (**h-i**) Identification  
882 of the genes differentially expressed in *Runx1/Foxl2* DKO vs. *Foxl2* KO ovaries and  
883 validation of candidate genes by quantitative PCR. (**j**) Expression of *Sox9* and *Amh* is  
884 not changed in *Runx1/Foxl2* DKO compared to *Foxl2* KO ovaries. For all the qPCR  
885 data, values are presented as mean  $\pm$  s.e.m (n=5/genotype). One-way ANOVA,  $P <$   
886 0.05. Bars with different letters (a, b, c) are significantly different.

887

888 **Figure 8: RUNX1 and FOXL2 exhibit extensive overlaps in chromatin binding in**  
889 **fetal ovaries.**

890 (**a**) *de novo* motif analysis of FOXL2 peaks identifies enrichment of RUNX motif along  
891 with FOXL2 motif in E14.5 ovaries. (**b**) The top *de novo* motif for RUNX1 ChIP-seq in  
892 E14.5 ovaries corresponds to a RUNX motif. (**c**) Distribution of genomic location of the  
893 10,494 RUNX1 binding peaks. TSS: Transcription Start Site; TES: Transcription End  
894 Site. (**d**) Comparison of RUNX1 (10,494 peaks) and FOXL2 (11,438 peaks) chromatin  
895 occupancy in E14.5 ovaries.

896

897 **Figure 9: Identification of the genes significantly changed in *Runx1/Foxl2* DKO**  
898 **that are nearest to RUNX1 and/or FOXL2 genomic binding peaks**

899 **(a)** Pie-chart of the genes significantly changed in *Runx1/Foxl2* DKO based on the  
900 presence of peak for FOXL2 and/or RUNX1. Genome browser view of 2 key genes  
901 significantly changed in *Runx1/Foxl2* DKO ovaries and bound by RUNX1 and FOXL2 in  
902 E14.5 ovaries. Blue arrows: gene orientation; Orange highlighted area: significant  
903 binding peaks identified by HOMER. **(b-d)** Examples of genes affected in *Runx1/Foxl2*  
904 DKO ovary and bound by FOXL2 or/and RUNX1. For each gene, we show the genome  
905 browser view of RUNX1 and/or FOXL2 binding in E14.5 ovaries, the gene expression  
906 by quantitative PCR in *Runx1/Foxl2* single and double knockouts at E15.5  
907 (n=4/genotype; mean  $\pm$  s.e.m. ; One-way ANOVA,  $P < 0.05$ .), and the gene expression  
908 in fetal Sertoli and granulosa cells from E11.5 to E13.5<sup>39</sup>. Bars with different letters (a,  
909 b) are significantly different.

**Figure 1:**

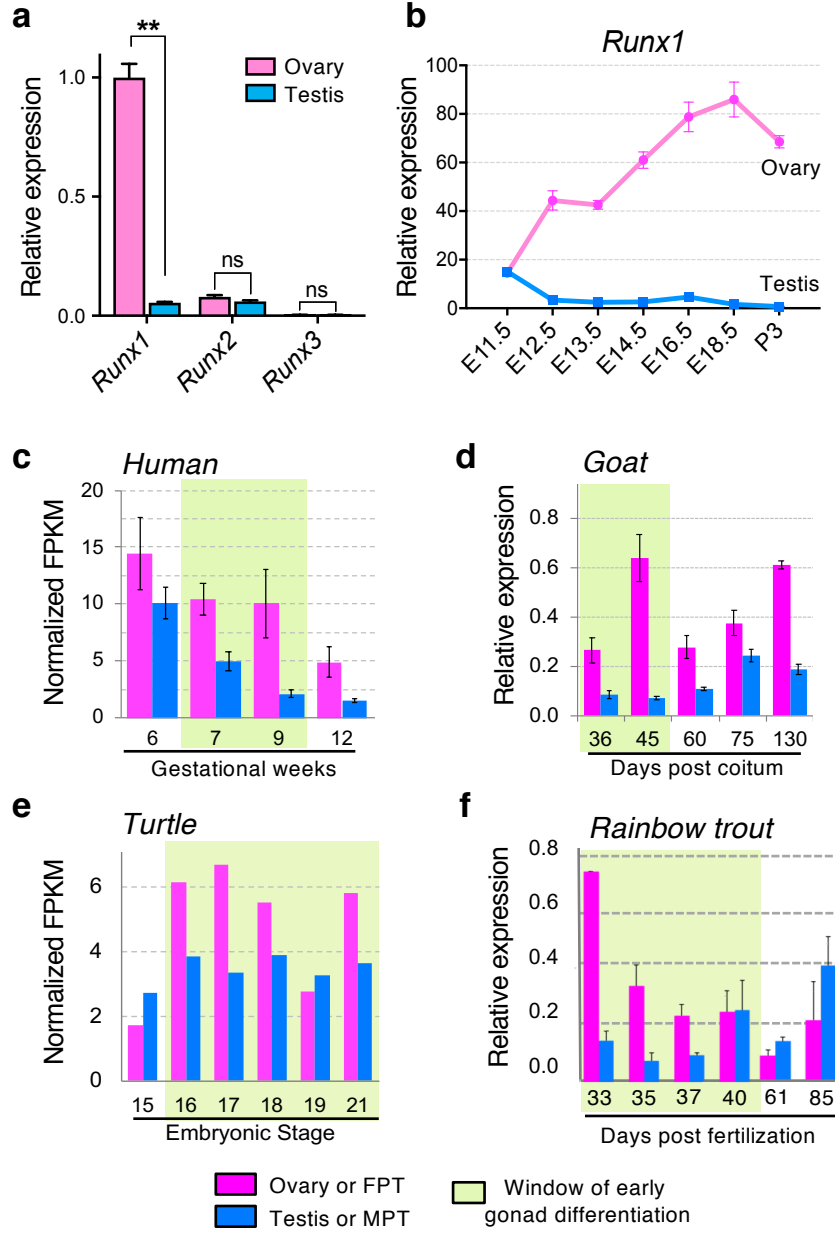


Figure 2

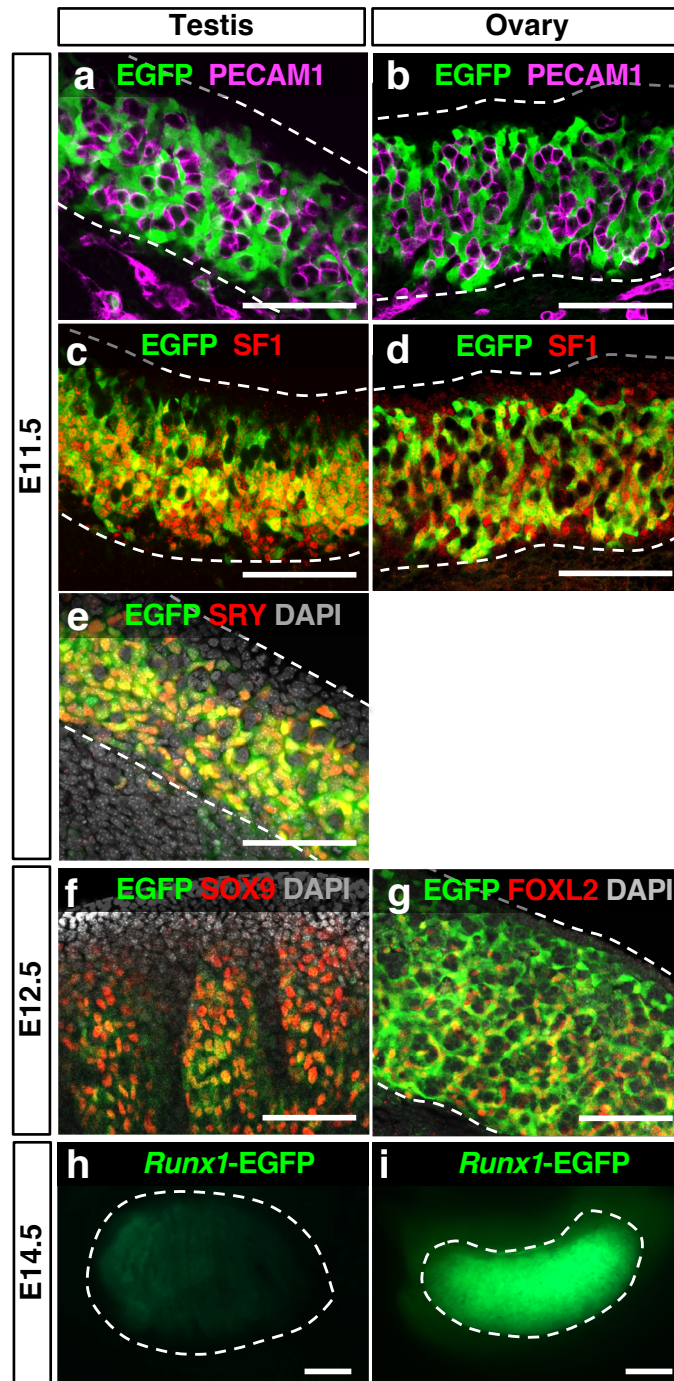
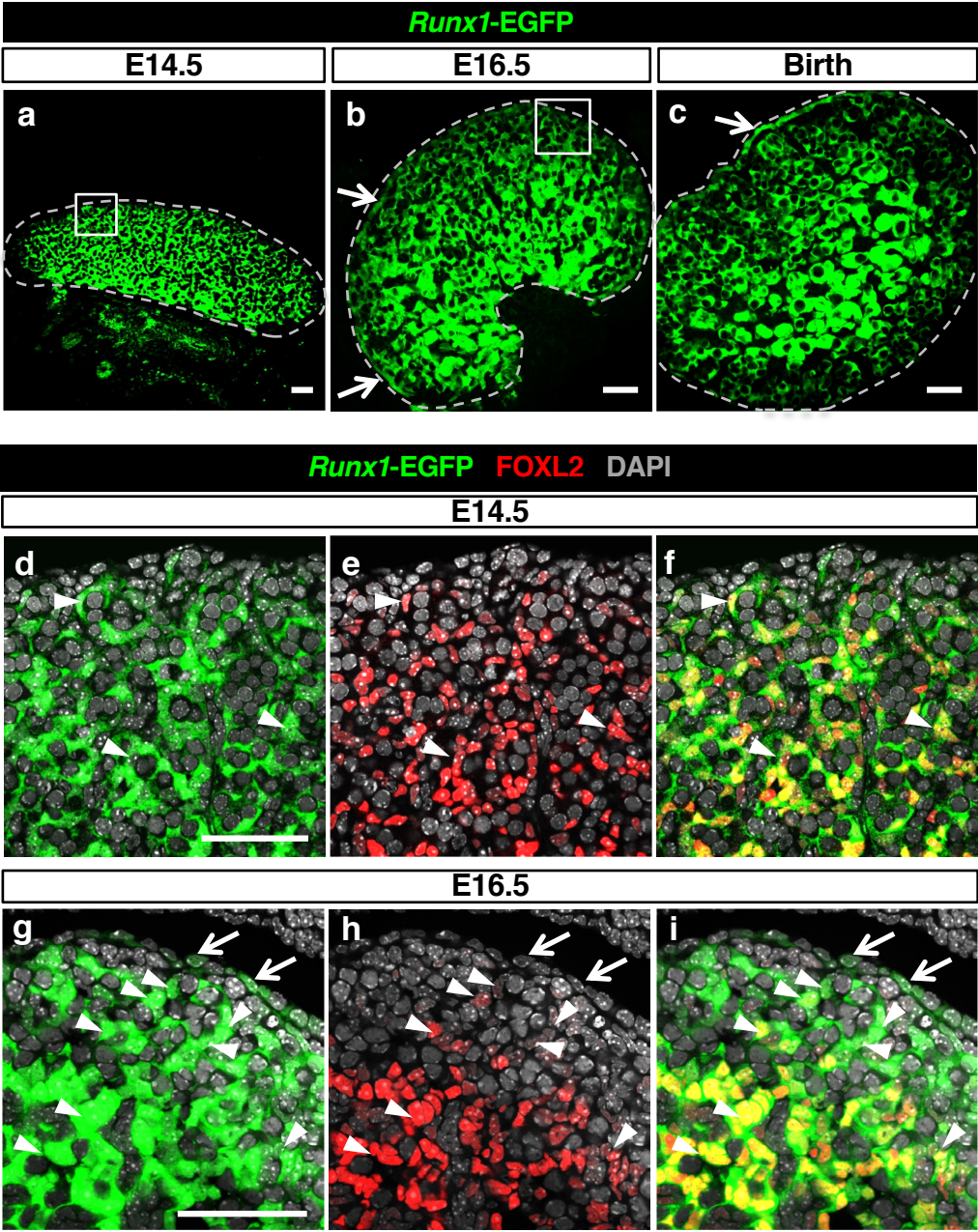




Figure 3:



**Figure 4:**

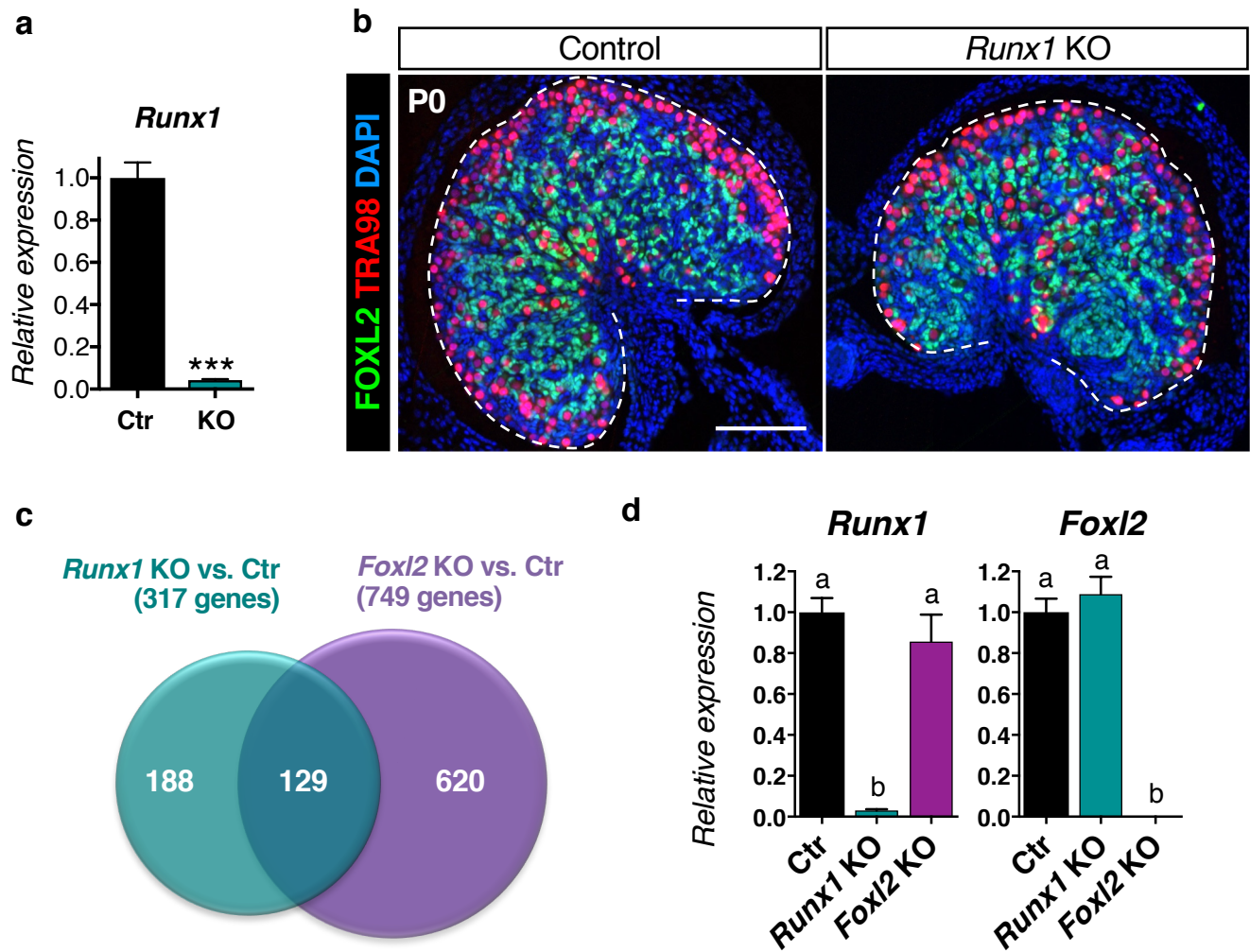


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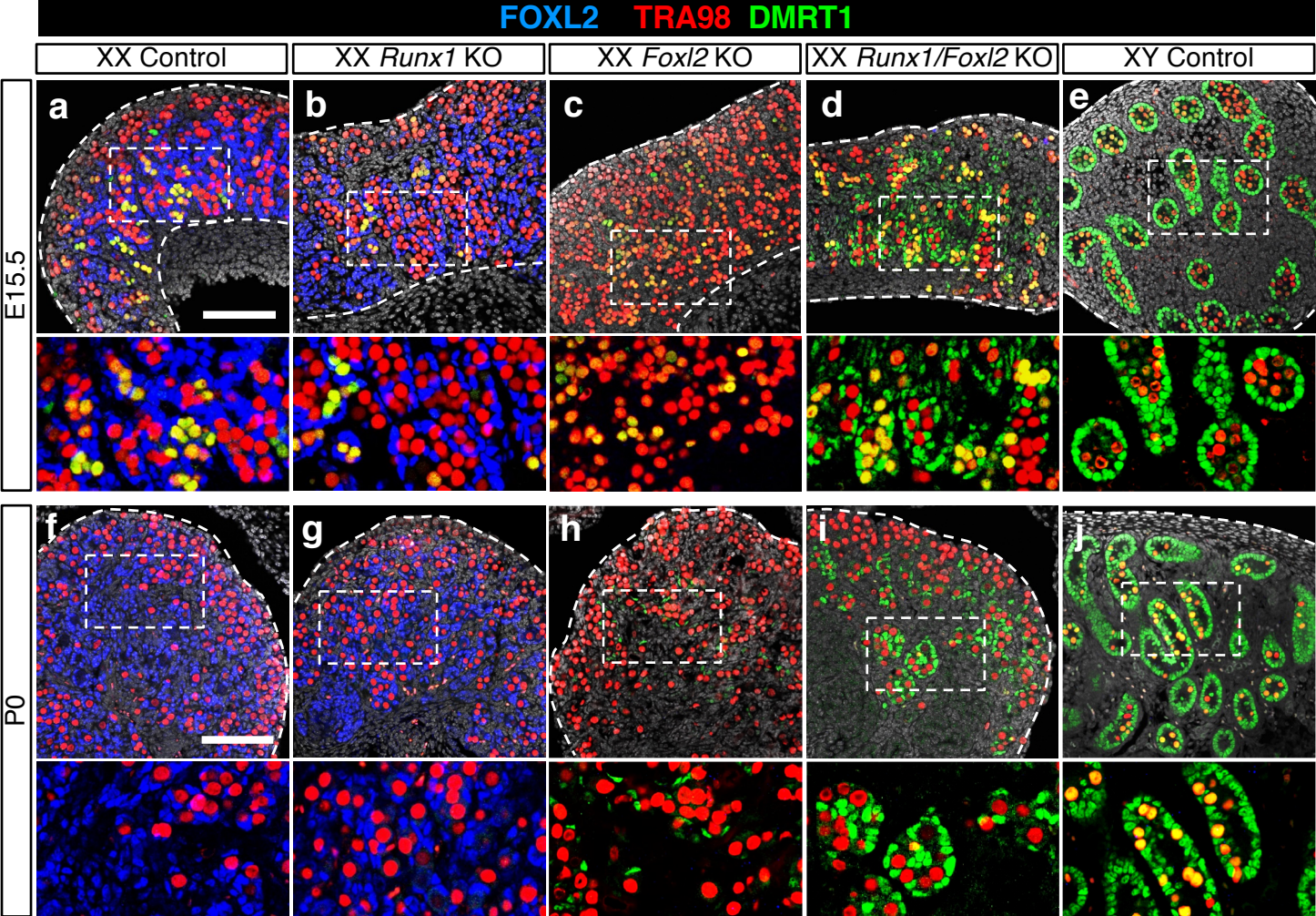
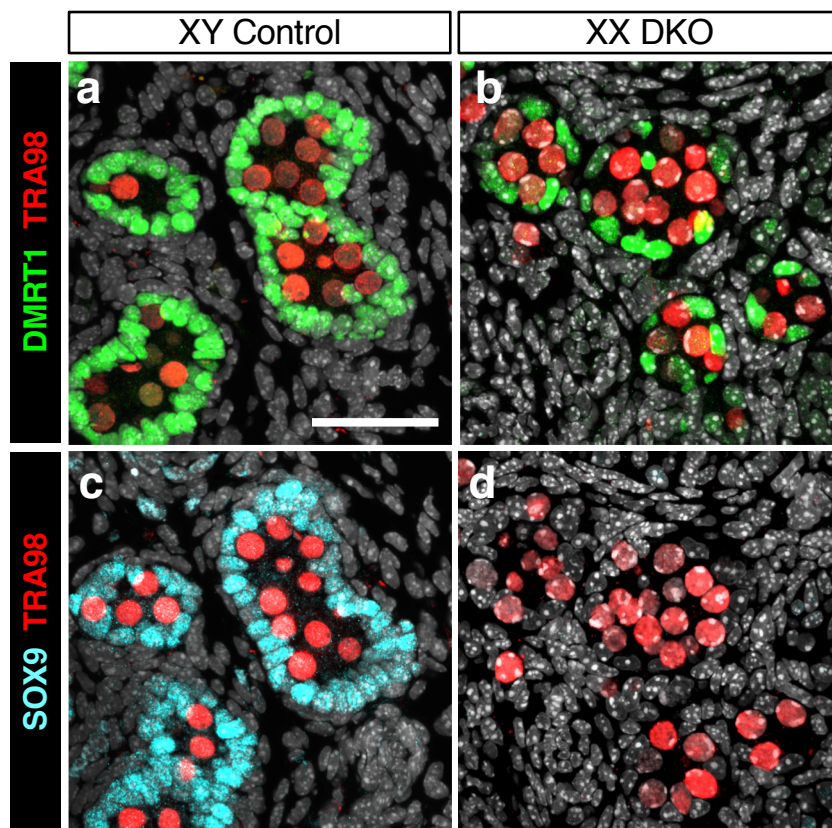


Figure 6:



**Figure 7:**

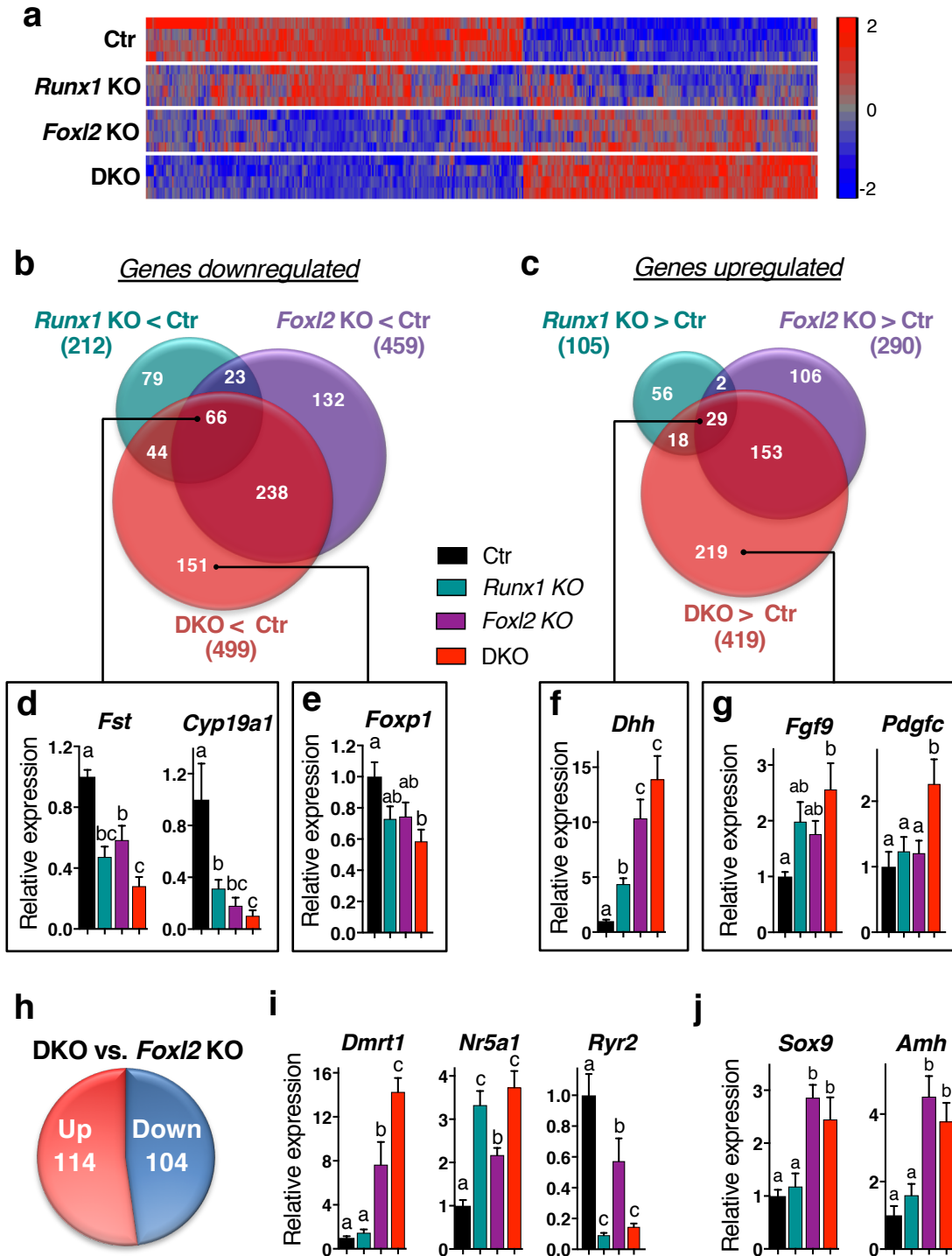
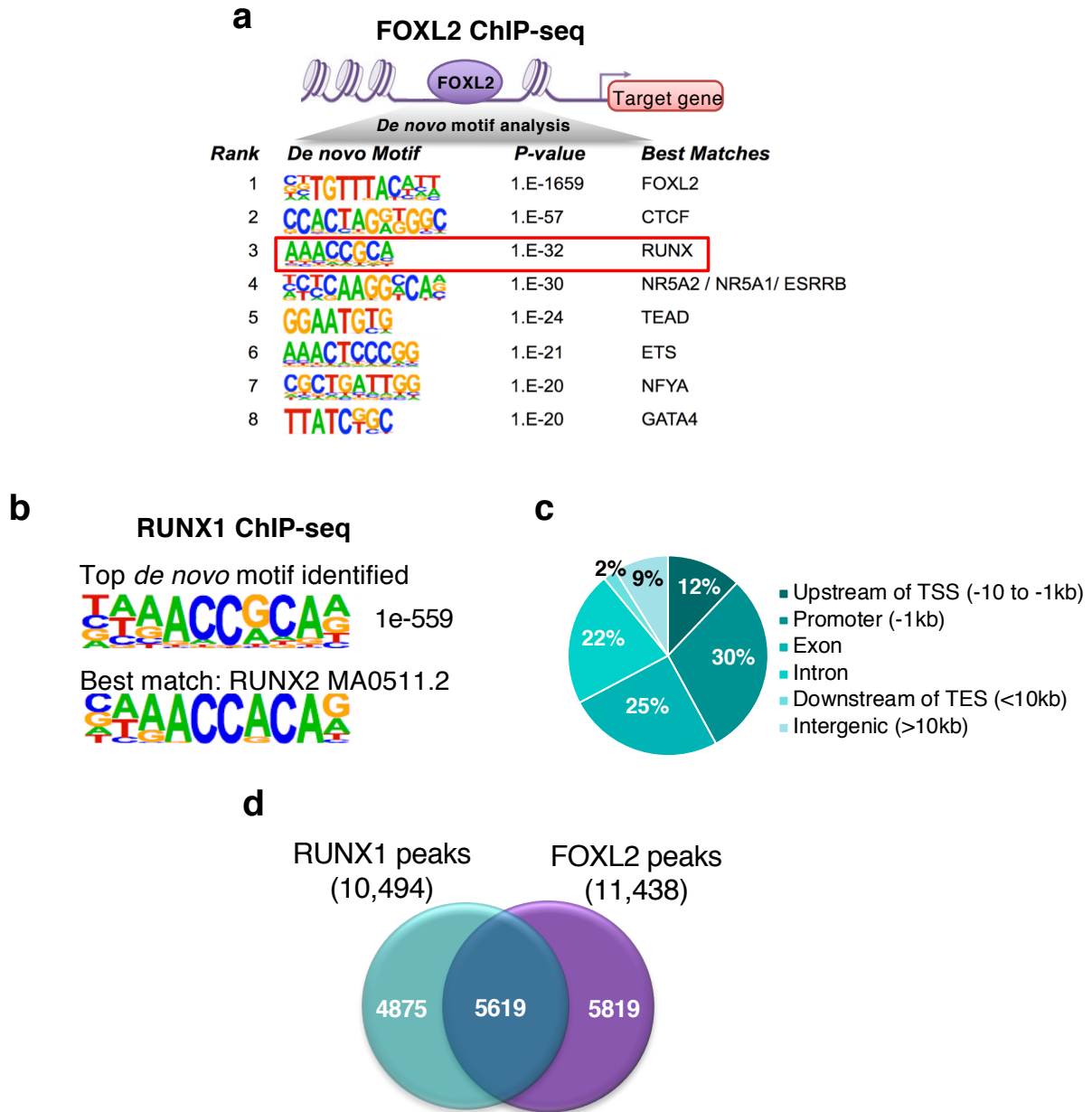


Figure 8:



**Figure 9:**

