

1 **Running head:** Phosphorylation by LATS1 regulates FOXL2 activity

2

3 **LATS1 phosphorylates Forkhead L2 and regulates its transcriptional activity**

4

5 *Margareta D. Pisarska^{1,2}, *Fang-Ting Kuo¹, Ikuko K. Bentsi-Barnes¹, Salma Khan¹, Gillian M.
6 Barlow¹

7

8 ¹Center for Fertility and Reproductive Medicine, Division of REI, Department of Ob/Gyn,
9 Cedars-Sinai Medical Center, Los Angeles, CA; ²David Geffen School of Medicine, UCLA, Los
10 Angeles, CA.

11 * These authors contributed equally to this work

12

13 **Corresponding author:**

14 Margareta D. Pisarska, M.D.

15 Center for Fertility and Reproductive Medicine, Dept. of Ob/Gyn, Cedars-Sinai Medical Center,
16 8635 West Third Street, Suite 160W, Los Angeles, CA 90048.

17 Phone: 310-423-5763; Fax: 310-423-0140

18 E-mail: pisarskam@cshs.org

19

20 **Abstract**

21 Forkhead L2 (FOXL2) is expressed in the ovary and acts as a transcriptional repressor of
22 the Steroidogenic Acute Regulatory (StAR) gene, a marker of granulosa cell differentiation.
23 Human FOXL2 mutations that produce truncated proteins lacking the C-terminus result in
24 blepharophimosis/ptosis/epicanthus inversus (BPES) syndrome type I, associated with premature
25 ovarian failure. In this study, we investigated whether FOXL2's activity as a transcriptional
26 repressor is regulated by phosphorylation. We found that FOXL2 is phosphorylated at a serine
27 residue and, using yeast two-hybrid screening, identified LATS1 as a potential FOXL2-
28 interacting protein. LATS1 is a serine/threonine kinase whose deletion in mice results in an
29 ovarian phenotype similar to POF. Using co-immunoprecipitation and kinase assays, we
30 confirmed that LATS1 binds to FOXL2, and demonstrated that LATS1 phosphorylates FOXL2
31 at a serine residue. Moreover, we found that FOXL2 and LATS1 are co-expressed in
32 developing mouse gonads and in granulosa cells of small and medium follicles in the mouse
33 ovary. Lastly, we demonstrated that co-expression with LATS1 enhances FOXL2's activity as a
34 repressor of the StAR promoter, and this results from the kinase activity of LATS1. These
35 results provide novel evidence that FOXL2 is phosphorylated by LATS1 and that this
36 phosphorylation enhances the transcriptional repression of the StAR gene, a marker of granulosa
37 cell differentiation. These data support our hypothesis that phosphorylation of FOXL2 may be a
38 control mechanism regulating the rate of granulosa cell differentiation and hence follicle
39 maturation, and its dysregulation may contribute to accelerated follicular development and
40 premature ovarian failure in BPES type I.

41 **Keywords:** Phosphorylation, transcriptional regulation, Forkhead, granulosa cell, premature
42 ovarian failure

43 **Introduction**

44 Premature ovarian failure (POF) is defined as a condition causing amenorrhea,
45 hypoestrogenism and elevated gonadotropins in women under 40 years of age (1), and can be
46 associated with failure to endow the follicle pool or an early loss of the fixed follicle pool
47 following excess follicle recruitment and/or atresia. A genetic basis for selective cases of POF
48 has been determined. Patients with Blepharophimosis-Ptoxis-Epicanthus Inversus (BPES)
49 syndrome type I exhibit POF in association with characteristic eyelid dysplasia,
50 blepharophimosis, ptoxis, and epicanthus inversus (60). Ovaries from BPES type I patients are
51 histologically variable, ranging from the presence of some primordial follicles with atretic
52 follicles to complete absence of follicles and scarring of the ovaries (23). The gene encoding the
53 transcription factor Forkhead L2 (FOXL2) (15) maps to the BPES locus on chromosome 3q22-
54 23. FOXL2 is a member of the forkhead/hepatocyte nuclear factor 3 (FKH/HNF3) family of
55 transcription factors (15), which is characterized by the presence of a conserved winged helix
56 domain that is essential for DNA binding, as well as more divergent transactivation or
57 transrepression domains (12, 29, 33). FOXL2 mutations in individuals with BPES type I create
58 premature stop codons which are predicted to generate truncated proteins lacking the carboxyl
59 (C)-terminus alanine/proline rich domain (15-17, 44). Consistent with the BPES phenotype,
60 FOXL2 is selectively expressed in the eyelids of developing mice (15) and in the mouse ovary
61 (41). In addition, FOXL2 shows a highly specific expression pattern in undifferentiated
62 granulosa cells of small and medium follicles in the mouse ovary, but is not expressed in the
63 other steroidogenic cells of the ovary, i.e. theca cells and luteal cells, nor is it expressed in non-
64 ovarian steroidogenic cells such as those of the testes or adrenal gland (15, 41).

65 We previously identified the C-terminal alanine/proline rich region of FOXL2 as a

66 transrepression domain (41). Further, we showed that a human mutation which is associated with
67 BPES type I produces a truncated FOXL2 protein that lacks this entire transrepression domain,
68 and loses its activity as a repressor of the StAR gene (41). StAR is a cholesterol transporter at the
69 mitochondrial membrane, and controls the rate-limiting step in steroidogenesis (14, 34, 49). It is
70 present in the granulosa cell layer of large preovulatory and luteinized follicles and absent from
71 immature follicles in several species (43, 46, 50). StAR activity is present in granulosa cells
72 during follicular differentiation, after FOXL2 expression decreases, signaling early functional
73 maturation of ovarian antral follicles (41, 46, 50). Thus, FOXL2 may inhibit premature
74 differentiation of granulosa cells and control the number of primordial follicles that remain
75 dormant, and mutations in FOXL2 may result in accelerated differentiation of granulosa cells,
76 ultimately leading to POF.

77 A number of forkhead transcription factors have been shown to be regulated through
78 phosphorylation, including members of the FOXO subfamily, which includes FOXO1, FOXO3
79 and FOXO4 (51). Outside the FOXO class, little is known about the regulation of other forkhead
80 transcription factors via phosphorylation. As premature follicle depletion is central to POF, we
81 hypothesized that more refined regulatory processes may be involved in controlling FOXL2
82 activity. In this study, we tested whether FOXL2 is regulated through phosphorylation, in order
83 to begin to define the mechanisms underlying granulosa cell differentiation, follicle maturation,
84 and premature ovarian failure.

85

86 **Materials and Methods**

87 *Yeast two-hybrid screening*

88 Yeast-two hybrid screening was performed using human FOXL2 as bait, and plasmids from

89 positive colonies were isolated, purified and sequenced (31). The specific interaction between
90 wild-type FOXL2 and LATS1 was confirmed based on activation of the GAL1-HIS3 reporter
91 gene.

92 ***Plasmid construction***

93 A FLAG-tagged FOXL2 cDNA was generated as described previously (31). A 3.4 kb human
94 LATS1 cDNA sequence was PCR-amplified from human ovary cDNA (Ambion, Austin, TX)
95 using the primers 5'-GTGGCGGCCCGCATGAAGAGGAGTGAAAA-3' and 5'-
96 TCGCGATCTAGTATATGTTTAACTCGAGTGA-3', and subcloned into pcDNA3 and
97 pcDNA3.1/His/Xpress expression vectors using the Not I and Xho I restriction sites to generate
98 LATS1 expression constructs. To generate the kinase-inactive LATS1 mutant D846A (13, 26), a
99 residue in the LATS1 kinase domain, Asp 846, was mutated to Ala, by performing site-directed
100 mutagenesis using this pcDNA3.1/His/Xpress-LATS1 construct and the primer 5'-
101 GGTCATATTAAATTGACTGCCTTTGGCCTCTGCACTGG-3'. The results were confirmed
102 by sequencing.

103 ***Cell culture and DNA transfection***

104 Chinese hamster ovary (CHO) cells and FOXL2 stable CHO cells were grown in culture and
105 transfected as described previously (31). The cells were then lysed using RIPA buffer, Tris
106 buffer (50mM Tris-HCl, 150mM sodium chloride, pH 7.4), immunoprecipitation buffer, or
107 reporter lysis buffer (Promega, Madison, WI) for subsequent experiments.

108 ***Alkaline phosphatase and phosphatase inhibitor treatments***

109 Cells were lysed with Tris buffer, heated at 95°C for 5 minutes to denature the proteins, and
110 the cell lysates (60 µg) were resuspended in de-phosphorylation buffer (50mM Tris-HCl, 0.1mM
111 EDTA, pH 8.5). The cell lysates were then treated with either (i) Halt Phosphatase Inhibitor

112 cocktail (Pierce, Rockford, IL); (ii) Halt Phosphatase Inhibitor cocktail and 5U alkaline
113 phosphatase (Roche, Indianapolis, IN), or (iii) 5U alkaline phosphatase alone. The cell lysates
114 were then incubated at 37°C for 1 hour, heated at 95°C for 15 minutes to inactivate the alkaline
115 phosphatase, and either analyzed directly by Western blotting, or immunoprecipitated (see
116 below) and then analyzed by Western blotting.

117 ***Immunoprecipitation***

118 For FOXL2, CHO cells were transfected with the pFLAG-FOXL2 expression construct for
119 24 hours in serum-free or normal culture media and then lysed and immunoprecipitated as
120 described previously (31). For negative controls, the cell lysates were immunoprecipitated with
121 Protein A (Millipore, Billerica, MA) coated with mouse IgG (Sigma, St. Louis, MO). The eluted
122 samples or cell lysates were added to 4x SDS sample buffer and heated at 95°C for 5 minutes to
123 denature the proteins. The proteins were then analyzed by Western blotting.

124 For LATS1, CHO cells were transfected with the pcDNA3.1-His-Xpress-LATS1 expression
125 construct for 24 hours and then lysed in immunoprecipitation buffer containing 50 mM Tris-HCl,
126 pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 0.2 mM PMSF. The cell lysates
127 were incubated with Protein A (Millipore, Billerica, MA) coated with LATS1 antibody (Bethyl
128 Laboratories, Montgomery, TX) or Xpress antibody (Invitrogen, Carlsbad, CA) at 4°C overnight.
129 The gel was washed with TBS (50 mM Tris-HCl, pH 7.4 and 150 mM NaCl) to eliminate non-
130 specific binding. The bound LATS1 was then eluted by incubating with 0.1M glycine HCl at pH
131 3.5 for 5 minutes. The supernatants were neutralized by adding 10X TBS and then used in the
132 kinase assays.

133 ***Kinase assays***

134 The pFLAG-FOXL2 expression construct was transfected into CHO cells. The lysates were

135 treated with alkaline phosphatase and the resulting de-phosphorylated FLAG-tagged FOXL2
136 proteins were purified by immunoprecipitation as described above. The pcDNA3.1-His-Xpress-
137 LATS1 expression construct was transfected into CHO cells in normal culture media and
138 purified using Protein A coated with LATS1 antibody or Xpress antibody, also as described
139 above. The purified FLAG-tagged FOXL2 proteins were then combined with the
140 immunoprecipitates containing wild type or mutant His-Xpress-LATS1, and the mixtures were
141 adjusted to 50mM Tris-HCl, 150mM sodium chloride, 2mM ATP, 10mM magnesium chloride,
142 10mM manganese chloride and incubated at 30°C for 4 hours. The protein/gel mixtures were
143 then added to 4x SDS sample buffer and heated at 95°C for 5 minutes to denature the proteins.
144 The assay products were then analyzed by Western blotting.

145 ***Western blotting***

146 Cells lysates in RIPA buffer, immunoprecipitation products, or kinase assay products were
147 added to 4x SDS sample buffer and heated at 95°C for 5 minutes to denature the proteins. The
148 proteins were then separated on 7.5% or 12% SDS-PAGE gels and transferred to PVDF
149 membranes. The membranes were incubated with a custom-made FOXL2 antibody (Zymed
150 Laboratories, San Francisco, CA), which was raised against amino acids 20-33 of FOXL2, or
151 with antibodies to LATS1 (Bethyl Laboratories, Montgomery, TX) or phosphoserine (Sigma),
152 washed, and then incubated with HRP-conjugated secondary antibodies. Chemiluminescent
153 detection was performed using ECL western blotting detection reagents (Amersham Biosciences,
154 Piscataway, NJ).

155 ***RNA extraction and reverse transcriptase PCR***

156 Mouse ovaries from fetal (17.5 days postconception), immature (day 13 and 23), and adult (7
157 weeks) mice were harvested from Swiss Webster outbred female mice under an approved

158 IACUC protocol. Total RNA from whole ovaries were isolated using an RNeasy mini kit
159 (Qiagen, Valencia, CA), and reverse transcribed to generate ovary cDNAs using an iScript
160 cDNA Synthesis Kit (Bio-Rad, Hercules, CA). A LATS1 cDNA sequence was then PCR
161 amplified from these ovary cDNAs using the primers 5'-TGCAGGAAATTCGAAACTCTC
162 TGCTTC-3' and 5'-GTGAGTTGGGACTCTCAGAATGATAGGCCA-3'. FOXL2 and GAPDH
163 primers were used as previously described (41). Human Ovary PCR-Ready cDNA (Ambion,
164 Austin, TX) was used as a positive control, and water was used as a negative control. After PCR,
165 the amplified products were subjected to electrophoresis on 1.5% agarose gels, which were then
166 stained with ethidium bromide to visualize the anticipated fragments. Bands of the expected
167 sizes were eluted, purified using a QIAquick gel extraction kit (Qiagen) and confirmed by
168 sequencing.

169 ***Immunohistochemistry***

170 Ovaries were dissected from immature Swiss Webster outbred female mice at 21 day of age
171 (under an approved IACUC protocol) and slides were prepared, pretreated and blocked as
172 described previously (31). The slides were then hybridized without primary antibody as a
173 control, or with a custom-made FOXL2 antibody (Zymed Laboratories) or with a LATS1
174 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour, followed by incubation with a
175 biotinylated anti-rabbit secondary antibody (Vector) for another 30 minutes. The slides were
176 then incubated with ABC solution (Vector) for 30 minutes and stained by adding fresh DAB
177 solution for 2 minutes. They were then counterstained with haematoxylin (Sigma) and mounted
178 with Vectamount AQ (Vector).

179 ***Promoter activity assays***

180 CHO cells or FOXL2 stable cells (31) were transfected with 100 ng of the human

181 pcDNA3.1-His-Xpress-LATS1 expression construct (wild type or D846A mutant) or the
182 pcDNA3.1/His vector backbone with 0.5 μ g of the 95 bp human StAR promoter luciferase
183 construct (41) for 24 hours. The indicator plasmid pCMV- β -galactosidase was used to estimate
184 transfection efficiency. The total DNA concentration was maintained at 1 μ g per well in 24 well
185 plate through the inclusion of empty pFLAG-CMV-2. Twenty-four hours after transfection, the
186 cells were lysed using reporter lysis buffer and repeated freeze/thaw cycles. The luciferase
187 activities in the cell lysates were determined as described previously (31). Results are reported
188 as relative luminescence units and normalized with β -galactosidase activity.

189 *Statistical Analysis*

190 For promoter assay studies, experiments were performed in quadruplicate and the standard
191 error (\pm SE) was shown. Significant differences between groups were analyzed by ANOVA
192 followed by the Neuman-Keuls test using GraphPad Prism software (San Diego, CA). *P* values <
193 0.05 were considered significant.

194

195 **Results**

196 *FOXL2 interacts with the serine/threonine kinase LATS1*

197 To identify FOXL2-interacting proteins, we performed a yeast two-hybrid screen using
198 human FOXL2. LATS1, a serine/threonine kinase, was found to strongly interact with FOXL2,
199 which suggested that LATS1 might be involved in phosphorylating FOXL2.

200 To characterize the interaction between FOXL2 and LATS1 in mammalian cells, CHO cells
201 were transfected with FLAG-FOXL2 expression construct or an empty FLAG-CMV-2
202 expression vector, and the cells were subsequently lysed and immunoprecipitated with an
203 antibody to FLAG, or with mouse IgG as a control. The cell lysates and immunoprecipitates

204 were then analyzed by immunoblotting with antibodies to FOXL2 and LATS1. LATS1 is
205 expressed endogenously in CHO cells, whereas FOXL2 is not. When the empty pFLAG-CMV-2
206 vector was used as a template for protein synthesis, no FOXL2 was synthesized, but when the
207 pcDNA3-FOXL2 construct was used as a template, FOXL2 was synthesized in the CHO cells
208 (Lysates, Fig. 1). Some endogenous LATS1 expression was also detected in the CHO cell lysates
209 (Lysates, Fig. 1). When the lysates from these cells were immunoprecipitated with the control
210 mouse IgG, a faint band was obtained for FLAG-FOXL2 in the immunoprecipitates from cells
211 expressing FLAG-FOXL2, but not in immunoprecipitates from cells expressing the empty
212 pFLAG-CMV-2 vector, and no band was obtained for LATS1 (IP: Mouse IgG, Fig.1). In
213 contrast when the same lysates were immunoprecipitated with an antibody to FLAG, both
214 FLAG-FOXL2 and LATS1 were identified in the immunoprecipitates from cells expressing
215 FLAG-FOXL2, but were not identified in immunoprecipitates from cells expressing the empty
216 pFLAG-CMV-2 vector (IP: FLAG, Fig. 1). These results confirm that FOXL2 and LATS1
217 interact with each other, as suggested by the results of the yeast two-hybrid screening.

218 ***FOXL2 is phosphorylated in CHO cells***

219 In order to test whether FOXL2 is phosphorylated in mammalian cells, we transfected CHO
220 cells (which do not express endogenous FOXL2) with the pcDNA3-FOXL2 expression construct
221 or with an empty pcDNA3 expression vector, lysed the cells under various conditions and
222 analyzed the resulting proteins by immunoblotting with the FOXL2 antibody (Fig. 2A). When
223 FOXL2-transfected cells were lysed in the presence of a phosphatase inhibitor to eliminate
224 phosphatase activity, two bands were detected (PI, Fig. 2A). The lower band was the same size
225 as the FOXL2 band previously detected (41), and the larger band was presumed to be
226 phosphorylated FOXL2. To confirm that this larger band was in fact phosphorylated FOXL2,

227 FOXL2-transfected cell lysates were treated with alkaline phosphatase to remove phospho-
228 groups. After phosphatase treatment, the larger FOXL2 band was eliminated (AP, Fig. 2A).
229 When the cell lysates were treated with both alkaline phosphatase and phosphatase inhibitor, the
230 larger band was preserved (AP+PI, Fig. 2A).

231 In order to determine whether FOXL2 is phosphorylated at a serine or a threonine residue,
232 CHO cells were transiently transfected with the pFLAG-FOXL2 expression construct or an
233 empty pFLAG-CMV-2 vector backbone, lysed, and cell lysates were immunoprecipitated using
234 an antibody to FLAG or mouse IgG. The immunoprecipitates were then analyzed by
235 immunoblotting with the FOXL2 antibody and a phospho-serine antibody (Figure 2B). When
236 the immunoprecipitates were probed with the FOXL2 antibody, the two bands we previously
237 identified corresponding to FOXL2 and phosphorylated FOXL2 respectively (Figure 2A) were
238 again seen in the FLAG immunoprecipitates, but not in the mouse IgG precipitates (Figure 2B).
239 When this blot was stripped and reprobed with a phospho-serine antibody, the upper band was
240 again identified, suggesting that this band is phospho-serine FOXL2 (pSerine-FOXL2) (Figure
241 2B). To further confirm these results, immunoprecipitates from cell lysates treated with alkaline
242 phosphatase were also immunoblotted with the FOXL2 and phospho-serine antibodies. When
243 the immunoprecipitates were probed with the FOXL2 antibody, a single band corresponding to
244 FOXL2 was seen in the FLAG immunoprecipitates, but not in the mouse IgG precipitates
245 (Figure 2C). When this blot was stripped and reprobed with the phospho-serine antibody, no
246 bands were observed (Figure 2C). No bands were detected when the immunoprecipitates were
247 probed with an anti-phospho-threonine antibody (data not shown). Taken together, these results
248 suggest that FOXL2 is phosphorylated at a serine residue in mammalian cells.

249

250 *LATS1 phosphorylates FOXL2 in vitro*

251 To determine whether FOXL2 is phosphorylated by LATS1, we performed a kinase assay in
252 which we used de-phosphorylated FOXL2 as a substrate for LATS1. The immunoprecipitates
253 containing LATS1 were then incubated with the de-phosphorylated FOXL2 for 4 hours at 30°C.
254 The products of the kinase reactions were then analyzed by immunoblotting with antibodies to
255 phospho-serine, FOXL2 and LATS1. As shown in Fig. 3A, in the absence of LATS1, FOXL2
256 was not phosphorylated at a serine residue, however, when de-phosphorylated FOXL2 was
257 incubated with increasing amounts of LATS1, a phospho-serine-FOXL2 band was observed in
258 the presence of LATS1 at the highest concentration (Fig. 3A), indicating that incubation with
259 LATS1 results in phosphorylation of FOXL2. These results suggest that LATS1 can act as a
260 kinase for FOXL2 *in vitro*.

261 To confirm that phosphorylation of FOXL2 is due primarily to the kinase activity of LATS1,
262 and not another protein kinase that co-purifies with LATS1, we repeated the kinase assays with
263 the inclusion of a kinase-inactive LATS1 mutant, D846A, which cannot bind ATP (13, 26).
264 CHO cells were transfected with pcDNA3.1-His-Xpress-LATS1 (wild type or mutant)
265 expression constructs, and lysates from these cells were purified using Protein A coated with
266 Xpress antibody. The immunoprecipitates containing wild type LATS1 or mutant LATS1
267 D846A were used to perform the kinase assays. A strong phospho-serine-FOXL2 band was
268 observed when wild-type LATS1 was used as the kinase, but only a very faint band was
269 observed when the D846A mutant LATS1 was used (Fig. 3B). These results indicate that
270 FOXL2 is primarily phosphorylated by LATS1.

271 *The LATS1 gene is expressed in developing mouse gonads*

272 Our previous studies showed that FOXL2 is expressed in the mouse ovary from at least 17.5

273 days postconception (dpc) to adulthood (41). As our results indicated that LATS1 can act as a
274 kinase for FOXL2 *in vitro*, we hypothesized if LATS1 also phosphorylates FOXL2 *in vivo*,
275 LATS1 and FOXL2 should have overlapping expression patterns in the mouse ovary. Therefore,
276 we used RT-PCR to determine LATS1 expression in fetal (17.5 dpc), immature (days 13 and 23)
277 and adult (7 weeks) mouse ovaries. As shown in Fig. 4, both FOXL2 and LATS1 are expressed
278 in the ovaries of fetal mice at 17.5 dpc, in the ovaries of immature mice at days 13 and 23 of life,
279 and in the ovaries of adult mice.

280 ***FOXL2 and LATS1 are co-expressed in granulosa cells in the mouse ovary***

281 The FOXL2 transcript has been shown to be specifically expressed in the granulosa cells of
282 small and medium follicles in the mouse ovary (41). To further investigate the respective
283 locations and expression levels of FOXL2 and LATS1 in the ovary, immunohistochemical
284 analysis was performed using mouse ovarian tissue sections. As the FOXL2 transcript is known
285 to be expressed in the less differentiated granulosa cells of small and medium follicles, serial
286 sections of ovaries from immature (21-day old) mice, which do not contain mature
287 (steroidogenic) follicles, were used for these studies. As shown in Fig. 5, FOXL2 and LATS1
288 were found to have overlapping patterns of expression in the granulosa cells of small (indicated
289 by arrows) and medium (indicated by arrowheads) follicles in the immature mouse ovary.

290 ***Co-expression with LATS1 enhances the repressive effect of FOXL2 on the StAR gene*** 291 ***promoter***

292 FOXL2 acts as a key transcriptional repressor during follicle development, and is known to
293 interact directly with the promoter of the StAR gene. In a previous study, we showed that
294 FOXL2 can repress the activity of a -95-bp human StAR promoter-luciferase construct in CHO
295 cells (41). Therefore, we used this construct to examine whether phosphorylation by LATS1 is

296 involved in regulating the repression of the StAR gene promoter by FOXL2. Wild-type CHO
297 cells and CHO cells expressing FOXL2 (31) were transfected with 100ng of the pcDNA3.1-His-
298 Xpress-LATS1 expression construct, 100 ng of the kinase-inactive D846A mutant LATS1
299 expression construct, or 100 ng of the empty pcDNA3.1-His vector backbone for 24 hours,
300 followed by co-transfection with the -95-bp human StAR promoter-luciferase reporter plasmid.
301 Twenty-four hours after transfection, the activity of the StAR promoter was assessed by
302 measuring luciferase activity in the cell lysates. As shown in Figure 6, in the absence of FOXL2,
303 luciferase activities in cells transfected with wild-type or mutant LATS1 were not significantly
304 different from cells transfected with the control vector (CHO cells, Fig. 6), indicating that
305 LATS1 itself does not affect StAR promoter activity. In contrast, in cells expressing FOXL2
306 (empty vector, FOXL2 stable cells), the basal level of StAR promoter activity was significantly
307 decreased ($p<0.05$), indicating that FOXL2 represses the activity of the StAR promoter, as
308 expected. When FOXL2-expressing cells were transfected with wild-type LATS1 (Wt LATS1,
309 FOXL2 stable cells), a further significant reduction in luciferase activity was observed ($p<0.05$),
310 indicating that StAR promoter activity was further repressed in the presence of both FOXL2 and
311 LATS1. In contrast, when FOXL2-expressing CHO cells were transfected with the kinase-
312 inactive LATS1 mutant, luciferase activity was restored to the levels seen in cells expressing
313 FOXL2 alone (Mt LATS1, FOXL2 stable cells). These results suggest that co-expression with
314 LATS1 enhances the activity of FOXL2 as a transcriptional repressor of the StAR gene, and that
315 this effect is due to the kinase activity of LATS1.

316

317 **Discussion**

318 In this manuscript, we have shown that the serine-threonine kinase LATS1 phosphorylates

319 FOXL2 *in vitro*, and that this phosphorylation occurs at a serine residue. Further, we have
320 demonstrated that LATS1 is expressed in the mouse ovary from at least 17.5 dpc through to
321 adulthood, and have localized this expression to the less differentiated granulosa cells of small
322 and medium follicles in the immature mouse ovary, where LATS1 is co-expressed with FOXL2.
323 Moreover, our data indicate that phosphorylation by LATS1 enhances, whereas a kinase-inactive
324 LATS1 mutant fails to enhance, FOXL2's activity as a transcriptional repressor of the StAR
325 gene, which controls a rate-limiting step in steroidogenesis and is a marker of granulosa cell
326 differentiation. Taken together, these data support our hypothesis that phosphorylation may be
327 one of the mechanisms controlling the activity of FOXL2 as a transcriptional repressor during
328 follicular development, and suggest that LATS1 may be involved in mediating this regulation.

329 Regulation through phosphorylation has been shown to be an important mechanism for
330 controlling the activities of transcription factors (28, 37), including other Forkhead transcription
331 factors (38) expressed in both human and rodent granulosa cells (42, 45). Protein kinase B
332 (PKB)/Akt phosphorylates the FOXO subfamily leading to export from the nucleus and
333 inhibition of transcriptional activities (6, 9, 30), whereas activation of the Ras-Ral pathway leads
334 to phosphorylation of FOXO members and induces transcriptional activity (18, 30). In rodent
335 granulosa cells, FOXO1 is phosphorylated through the PI3K pathway in response to Follicle
336 Stimulating Hormone (FSH) and Insulin-like Growth Factor 1 (IGF-I), leading to nuclear
337 exclusion (45). Moreover, key genes in the lipid, sterol, and steroidogenic biosynthetic pathways
338 which are induced by FSH are suppressed by constitutively active FOXO1, indicating that
339 FOXO1 functions as a transcriptional repressor (35). Similar to FOXO1, we have found that
340 FOXL2 also plays a key role in transcriptional repression of the rate limiting step of the
341 steroidogenic pathway (41). However, in contrast to the FOXO family, little has been identified

342 regarding the regulation of FOXL2 and other forkhead transcription factors via phosphorylation.
343 However, a study by Benayoun et al. (2), which was performed using the granulosa-like KGN
344 cell line, suggested that FOXL2 is highly modified post-translationally and that multiple
345 phosphorylation events were likely involved in its modification. Our results demonstrate for the
346 first time that FOXL2 is indeed phosphorylated, and that LATS1 is at least one kinase that is
347 involved in its phosphorylation and subsequent functional activity, as indicated by the increase in
348 FOXL2's activity as a transcriptional repressor of the StAR gene in the presence of wild-type,
349 but not mutant, LATS1.

350 We have previously demonstrated that FOXL2 is expressed in granulosa cells, where it may
351 be involved in regulating the early stages of folliculogenesis (41). We demonstrated that FOXL2
352 is expressed in granulosa cells of small and medium follicles in the immature mouse ovary, and
353 also in a few type 6 follicles (41). In larger follicles, FOXL2 is expressed in the less
354 differentiated cumulus cells, but shows much lower expression in mural granulosa cells (41). In
355 contrast to FOXL2, the StAR gene is not expressed in immature follicles (43, 46, 50), but is
356 present in granulosa cells of large preovulatory and luteinized follicles in humans, mural
357 granulosa cells in the rodent ovary following treatment with PMSG, and granulosa cells of
358 periovulatory follicles (43, 46, 50). Therefore, FOXL2 appears to repress StAR expression
359 during follicle development, and it is only with loss of this transcriptional repression that StAR
360 begins to be expressed in granulosa cells, signaling early functional maturation of ovarian antral
361 follicles. Further, FOXL2 may also work globally as a transcriptional repressor of granulosa cell
362 proliferation and differentiation, as demonstrated by its effects on the aromatase, P450scc, cyclin
363 D2 promoters (4) and the *CYP17* promoter (39). Interestingly, anti-mullerian hormone (AMH)
364 (20, 27, 54) and activin A (22, 55) are also expressed in granulosa cells of primary and growing

365 follicles and may function as inhibitory factors for the primordial to primary follicle transition (8,
366 11, 53). AMH and activin A are specifically downregulated in the ovaries of FOXL2 knockout
367 mice (47), which exhibit early follicular depletion and undergo primary ovarian failure (47).
368 Taken together, these data suggest that FOXL2 may function as a determinant of tissue
369 differentiation by inhibiting premature differentiation of granulosa cells, possibly acting through
370 AMH and/or activin A. With the inhibition of granulosa cell differentiation, FOXL2 may control
371 the number of primordial follicles that remain dormant and prevent the premature depletion of
372 ovarian follicles.

373 FOXL2 in the ovary is one of the first sexually dimorphic genes expressed and a
374 determinant of tissue differentiation. Likewise, FOXL2 in the pituitary also precedes the
375 expression of gonadotrope-specific markers and has been suggested to be involved in the
376 differentiation of this lineage (7, 21, 52). In the gonadotrope, FOXL2 functions as a SMAD 3
377 partner and drives transcription in α T3-1 cells treated with activin (7). Lamba et al (32) also
378 recently demonstrated that FOXL2 is involved in mediating activin A-regulated murine FSH β
379 (*Fshb*) transcription in pituitary gonadotropes, likely in association with SMAD3. These
380 findings suggest that FOXL2 functions as a transcriptional regulator and a coordinator of
381 SMAD3 targets in pituitary gonadotropes (7) and differentiation may be regulated by interaction
382 of the forkhead and SMAD family of transcription factors under the control of activin.

383 Activin, in conjunction with FSH also plays a significant role in granulosa cell proliferation
384 and differentiation, through phosphorylation. Phosphorylation of FOXO1 and SMAD3 by FSH
385 and activin leads to stimulation of granulosa cell differentiation and proliferation (40). Similarly,
386 we have demonstrated that FOXL2 is also phosphorylated, through LATS1, which is present in
387 granulosa cells of immature follicles, and this phosphorylation may enhance the activity of

388 FOXL2 as a transcriptional repressor which may regulate differentiation, perhaps with the
389 SMAD family of transcription factors, as demonstrated in other cell types of the reproductive
390 pathway, such as the gonadotropes (7, 32) .

391 LATS1 is part of the Hippo pathway, which was originally defined in *Drosophila* and is
392 conserved in mammals. LATS1 is a serine/threonine kinase with demonstrated tumor suppressor
393 activity in *Drosophila* (57), and has been shown to inhibit cell proliferation *in vitro* via its kinase
394 activity (56). Studies have shown that the Hippo pathway suppresses tumor growth by
395 regulating cell proliferation, growth and death (10, 19, 58, 59). The component proteins in
396 *Drosophila*, Hippo, Sav, Wts, and Mats, are conserved in mammals as Mst1/2, WW45,
397 LATS1/2, and Mob1 respectively. In mammals, LATS1 is regulated by the MOB superfamily
398 proteins - MOB1 associates with LATS1 and activates it, and MST2 phosphorylates and
399 activates LATS1 (5, 25). LATS1 in turn binds to and phosphorylates the transcriptional activator
400 YAP (24). LATS1 is highly expressed in the ovary (48) and we found LATS1 to be highly
401 expressed in the granulosa cells of small and medium follicles, cells that have the potential for
402 rapid proliferation. LATS1 knockout mice have severe fertility defects: their ovaries contain
403 fewer follicles than wild-types, with only primary or secondary follicles present. Although some
404 of the fertility defects are attributed to an isolated LH-hypogonadotropic hypogonadism, some
405 LATS1-deficient females are able to give birth to 1-3 litters before becoming infertile, and those
406 females who are initially fertile appear to undergo premature ovarian failure (48). These fertility
407 defects are reminiscent of those seen in women with BPES type 1 and heterozygous mutations of
408 FOXL2, who exhibit a complete sequence of follicle development to the ovulatory stage with
409 early depletion of the follicle pool and premature ovarian failure (23). In contrast, FOXL2 null
410 female mice exhibit a more severe ovarian phenotype, with follicular arrest between the

411 primordial and primary stages followed by follicle degeneration (47). They fail to undergo
412 sexual maturation, and undergo primary ovarian failure; thus, unlike the heterozygous mutation
413 in humans, the complete loss of FOXL2 in these mice may result in this more severe phenotype
414 and hence primary ovarian failure and not secondary failure as seen in BPES type 1.

415 Xia et al. (56) demonstrated that a kinase-inactive LATS1 mutant protein fails to induce
416 apoptosis of MCF-7 cells, and loses its ability to inhibit cell growth, suggesting that the kinase
417 activity of LATS1 is required for the induction of apoptosis and inhibition of cell proliferation
418 (56). Therefore, we hypothesize that LATS1 and FOXL2 may function in a common pathway
419 inhibiting proliferation and differentiation until follicle development is signaled. In this
420 manuscript, we have shown that co-expression with LATS1 enhances the ability of FOXL2 to
421 repress transcription of the StAR promoter, whereas co-expression with the kinase-inactive
422 LATS1^{D846A} mutant failed to enhance FOXL2's activity as a transcriptional repressor. These
423 results suggest that the kinase activity of LATS1 is involved in regulating FOXL2's functional
424 activity as a transcriptional repressor.

425 We found that LATS1 and FOXL2 are co-expressed in less differentiated granulosa cells of
426 small and medium ovarian follicles in the mouse ovary, and that LATS1 phosphorylates FOXL2
427 and enhances its transcriptional repression of the StAR gene *in vitro*. These data are consistent
428 with our hypothesis that LATS1 may phosphorylate FOXL2 during follicle development. We
429 propose that this phosphorylation may be one of the mechanisms by which FOXL2's activity as
430 a transcriptional repressor is regulated *in vivo*, and that release of repression may allow the StAR
431 gene to be expressed in granulosa cells, signaling granulosa cell differentiation and proliferation
432 and subsequent functional maturation of ovarian follicles. FOXL2 is also sumoylated (3, 36), and
433 sumoylation is also necessary for transcriptional regulation (31). Thus, it appears that there are

434 multiple fine tuning mechanisms necessary for FOXL2 as a transcriptional regulator, including
435 its potential role with the SMAD family of transcription factors as demonstrated in other cell
436 types (7). Whether there is a mechanism similar to FOXO1 cooperativity with the SMAD family
437 of transcription factors in granulosa cells (40) that leads to granulosa cell proliferation and
438 differentiation remains to be determined.

439

440 **Acknowledgements**

441 We thank J. F. Strauss III for the StAR promoter construct.

442

443 **Grants**

444 This work was supported by R01HD047603 from the National Institute of Child Health and
445 Human Development (NICHD) and the Office of Research on Women's Health (ORWH) (MP)
446 and by a grant from the Helping Hands of Los Angeles, Inc. (MP).

447

448 **References**

- 449 1. **Adashi EY.** Immune modulators in the context of the ovulatory process: a role for
450 interleukin-1. *Am J Reprod Immunol* 35: 190-194, 1996.
- 451 2. **Benayoun BA, Auer J, Caburet S, and Veitia RA.** The post-translational modification
452 profile of the forkhead transcription factor FOXL2 suggests the existence of parallel
453 processive/concerted modification pathways. *Proteomics* 8: 3118-3123, 2008.
- 454 3. **Benayoun BA, Caburet S, Dipietromaria A, Georges A, D'Haene B, Pandaranayaka PJ,**
455 **L'Hote D, Todeschini AL, Krishnaswamy S, Fellous M, De Baere E, and Veitia RA.**
456 Functional exploration of the adult ovarian granulosa cell tumor-associated somatic FOXL2
457 mutation p.Cys134Trp (c.402C>G). *PloS One* 5: e8789.
- 458 4. **Bentsi-Barnes IK, Kuo FT, Barlow GM, and Pisarska MD.** Human forkhead L2 represses
459 key genes in granulosa cell differentiation including aromatase, P450scc, and cyclin D2.
460 *Fertility and Sterility*, 2009.
- 461 5. **Bichsel SJ, Tamaskovic R, Stegert MR, and Hemmings BA.** Mechanism of activation of
462 NDR (nuclear Dbf2-related) protein kinase by the hMOB1 protein. *The Journal of Biological*
463 *Chemistry* 279: 35228-35235, 2004.

- 464 6. **Biggs WH, 3rd, Meisenhelder J, Hunter T, Cavenee WK, and Arden KC.** Protein kinase
465 B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix
466 transcription factor FKHR1. *Proceedings of the National Academy of Sciences of the United*
467 *States of America* 96: 7421-7426, 1999.
- 468 7. **Blount AL, Schmidt K, Justice NJ, Vale WW, Fischer WH, and Bilezikjian LM.** FoxL2
469 and Smad3 coordinately regulate follistatin gene transcription. *The Journal of Biological*
470 *Chemistry* 284: 7631-7645, 2009.
- 471 8. **Braw-Tal R.** The initiation of follicle growth: the oocyte or the somatic cells? *Molecular &*
472 *Cellular Endocrinology* 187: 11-18, 2002.
- 473 9. **Brunet A, Bonni A, Zigmund MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC,**
474 **Blenis J, and Greenberg ME.** Akt promotes cell survival by phosphorylating and inhibiting
475 a Forkhead transcription factor. *Cell* 96: 857-868, 1999.
- 476 10. **Camargo FD, Gokhale S, Johnnidis JB, Fu D, Bell GW, Jaenisch R, and Brummelkamp**
477 **TR.** YAP1 increases organ size and expands undifferentiated progenitor cells.[erratum
478 appears in *Curr Biol.* 2007 Dec 4;17(23):2094]. *Current Biology* 17: 2054-2060, 2007.
- 479 11. **Carlsson IB, Scott JE, Visser JA, Ritvos O, Themmen AP, and Hovatta O.** Anti-
480 Mullerian hormone inhibits initiation of growth of human primordial ovarian follicles in
481 vitro. *Human Reproduction (Oxford, England)* 21: 2223-2227, 2006.
- 482 12. **Carlsson P and Mahlapuu M.** Forkhead transcription factors: key players in development
483 and metabolism. *Developmental Biology* 250: 1-23, 2002.
- 484 13. **Chan EH, Nousiainen M, Chalamalasetty RB, Schafer A, Nigg EA, and Sillje HH.** The
485 Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene*
486 24: 2076-2086, 2005.
- 487 14. **Clark BJ, Wells J, King SR, and Stocco DM.** The purification, cloning, and expression of a
488 novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor
489 cells. Characterization of the steroidogenic acute regulatory protein (StAR). *The Journal of*
490 *Biological Chemistry* 269: 28314-28322, 1994.
- 491 15. **Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L,**
492 **Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-**
493 **Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, and**
494 **Pilia G.** The putative forkhead transcription factor FOXL2 is mutated in
495 blepharophimosis/ptosis/epicanthus inversus syndrome. *Nature Genetics* 27: 159-166, 2001.
- 496 16. **De Baere E, Beysen D, Oley C, Lorenz B, Cocquet J, De Sutter P, Devriendt K, Dixon**
497 **M, Fellous M, Fryns JP, Garza A, Jonsrud C, Koivisto PA, Krause A, Leroy BP, Meire**
498 **F, Plomp A, Van Maldergem L, De Paepe A, Veitia R, and Messiaen L.** FOXL2 and
499 BPES: mutational hotspots, phenotypic variability, and revision of the genotype-phenotype
500 correlation. *American Journal of Human Genetics* 72: 478-487, 2003.
- 501 17. **De Baere E, Dixon MJ, Small KW, Jabs EW, Leroy BP, Devriendt K, Gillerot Y,**
502 **Mortier G, Meire F, Van Maldergem L, Courtens W, Hjalgrim H, Huang S, Liebaers I,**
503 **Van Regemorter N, Touraine P, Praphanphoj V, Verloes A, Udard N, Yellore V,**
504 **Chalukya M, Yelchits S, De Paepe A, Kuttann F, Fellous M, Veitia R, and Messiaen L.**
505 Spectrum of FOXL2 gene mutations in blepharophimosis-ptosis-epicanthus inversus (BPES)
506 families demonstrates a genotype-phenotype correlation. *Human Molecular Genetics* 10:
507 1591-1600, 2001.

- 508 18. **De Ruiter ND, Burgering BM, and Bos JL.** Regulation of the Forkhead transcription factor
509 AFX by Ral-dependent phosphorylation of threonines 447 and 451. *Molecular and Cellular*
510 *Biology* 21: 8225-8235, 2001.
- 511 19. **Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders**
512 **RA, Maitra A, and Pan D.** Elucidation of a universal size-control mechanism in *Drosophila*
513 and mammals. *Cell* 130: 1120-1133, 2007.
- 514 20. **Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW,**
515 **Uilenbroek JT, Grootegoed JA, and Themmen AP.** Anti-Mullerian hormone inhibits
516 initiation of primordial follicle growth in the mouse ovary. *Endocrinology* 143: 1076-1084,
517 2002.
- 518 21. **Ellsworth BS, Egashira N, Haller JL, Butts DL, Cocquet J, Clay CM, Osamura RY,**
519 **and Camper SA.** FOXL2 in the pituitary: molecular, genetic, and developmental analysis.
520 *Molecular Endocrinology* 20: 2796-2805, 2006.
- 521 22. **Findlay JK.** An update on the roles of inhibin, activin, and follistatin as local regulators of
522 folliculogenesis. *Biology of Reproduction* 48: 15-23, 1993.
- 523 23. **Fraser IS, Shearman RP, Smith A, and Russell P.** An association among
524 blepharophimosis, resistant ovary syndrome, and true premature menopause. *Fertility and*
525 *Sterility* 50: 747-751, 1988.
- 526 24. **Hao Y, Chun A, Cheung K, Rashidi B, and Yang X.** Tumor suppressor LATS1 is a
527 negative regulator of oncogene YAP. *The Journal of Biological Chemistry* 283: 5496-5509,
528 2008.
- 529 25. **Hergovich A, Bichsel SJ, and Hemmings BA.** Human NDR kinases are rapidly activated
530 by MOB proteins through recruitment to the plasma membrane and phosphorylation.
531 *Molecular and Cellular Biology* 25: 8259-8272, 2005.
- 532 26. **Hergovich A, Schmitz D, and Hemmings BA.** The human tumour suppressor LATS1 is
533 activated by human MOB1 at the membrane. *Biochemical and Biophysical Research*
534 *Communications* 345: 50-58, 2006.
- 535 27. **Hirobe S, He WW, Lee MM, and Donahoe PK.** Mullerian inhibiting substance messenger
536 ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic
537 activity. *Endocrinology* 131: 854-862, 1992.
- 538 28. **Karin M and Hunter T.** Transcriptional control by protein phosphorylation: signal
539 transmission from the cell surface to the nucleus. *Curr Biol* 5: 747-757, 1995.
- 540 29. **Kaufmann E and Knochel W.** Five years on the wings of fork head. *Mechanisms of*
541 *Development* 57: 3-20, 1996.
- 542 30. **Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, and Burgering BM.**
543 Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* 398:
544 630-634, 1999.
- 545 31. **Kuo FT, Bentsi-Barnes IK, Barlow GM, Bae J, and Pisarska MD.** Sumoylation of
546 forkhead L2 by Ubc9 is required for its activity as a transcriptional repressor of the
547 Steroidogenic Acute Regulatory gene. *Cellular Signalling* 21: 1935-1944, 2009.
- 548 32. **Lamba P, Fortin J, Tran S, Wang Y, and Bernard DJ.** A novel role for the forkhead
549 transcription factor FOXL2 in activin A-regulated follicle-stimulating hormone beta subunit
550 transcription. *Molecular Endocrinology* 23: 1001-1013, 2009.
- 551 33. **Lehmann OJ, Sowden JC, Carlsson P, Jordan T, and Bhattacharya SS.** Fox's in
552 development and disease. *Trends Genet* 19: 339-344, 2003.

- 553 34. **Lin D, Sugawara T, Strauss JF, 3rd, Clark BJ, Stocco DM, Saenger P, Rogol A, and**
554 **Miller WL.** Role of steroidogenic acute regulatory protein in adrenal and gonadal
555 steroidogenesis. *Science (New York)* 267: 1828-1831, 1995.
- 556 35. **Liu Z, Rudd MD, Hernandez-Gonzalez I, Gonzalez-Robayna I, Fan H-Y, Zeleznik AJ,**
557 **and Richards JS.** FSH and FOXO1 regulate genes in the sterol/steroid and lipid biosynthetic
558 pathways in granulosa cells. *Molecular Endocrinology* 23: 649-661, 2009.
- 559 36. **Marongiu M, Deiana M, Meloni A, Marcia L, Puddu A, Cao A, Schlessinger D, and**
560 **Crisponi L.** The forkhead transcription factor Foxl2 is sumoylated in both human and
561 mouse: sumoylation affects its stability, localization, and activity. *PLoS One* 5: e9477.
- 562 37. **Montminy M.** Transcriptional activation. Something new to hang your HAT on. *Nature* 387:
563 654-655, 1997.
- 564 38. **Obsil T and Obsilova V.** Structure/function relationships underlying regulation of FOXO
565 transcription factors. *Oncogene* 27: 2263-2275, 2008.
- 566 39. **Park M, Shin E, Won M, Kim JH, Go H, Kim HL, Ko JJ, Lee K, and Bae J.** FOXL2
567 Interacts with Steroidogenic Factor-1 (SF-1) and Represses SF-1-Induced CYP17
568 Transcription in Granulosa Cells. *Molecular Endocrinology (Baltimore, MD)*.
- 569 40. **Park Y, Maizels ET, Feiger ZJ, Alam H, Peters CA, Woodruff TK, Unterman TG, Lee**
570 **EJ, Jameson JL, and Hunzicker-Dunn M.** Induction of cyclin D2 in rat granulosa cells
571 requires FSH-dependent relief from FOXO1 repression coupled with positive signals from
572 Smad. *The Journal of Biological Chemistry* 280: 9135-9148, 2005.
- 573 41. **Pisarska MD, Bae J, Klein C, and Hsueh AJ.** Forkhead l2 is expressed in the ovary and
574 represses the promoter activity of the steroidogenic acute regulatory gene. *Endocrinology*
575 145: 3424-3433, 2004.
- 576 42. **Pisarska MD, Kuo F-T, Tang D, Zarrini P, Khan S, and Ketefian A.** Expression of
577 forkhead transcription factors in human granulosa cells. *Fertility & Sterility* 91: 1392-1394,
578 2009.
- 579 43. **Pollack SE, Furth EE, Kallen CB, Arakane F, Kiriakidou M, Kozarsky KF, and Strauss**
580 **JF, 3rd.** Localization of the steroidogenic acute regulatory protein in human tissues. *The*
581 *Journal of Clinical Endocrinology and Metabolism* 82: 4243-4251, 1997.
- 582 44. **Ramirez-Castro JL, Pineda-Trujillo N, Valencia AV, Muneton CM, Botero O, Trujillo**
583 **O, Vasquez G, Mora BE, Durango N, Bedoya G, and Ruiz-Linares A.** Mutations in
584 FOXL2 underlying BPES (types 1 and 2) in Colombian families. *American Journal of*
585 *Medical Genetics* 113: 47-51, 2002.
- 586 45. **Richards JS, Sharma SC, Falender AE, and Lo YH.** Expression of FKHR, FKHL1, and
587 AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the
588 gonadotropins. *Molecular Endocrinology (Baltimore, MD)* 16: 580-599, 2002.
- 589 46. **Ronen-Fuhrmann T, Timberg R, King SR, Hales KH, Hales DB, Stocco DM, and Orly**
590 **J.** Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR)
591 during follicular development in the rat ovary. *Endocrinology* 139: 303-315, 1998.
- 592 47. **Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, and Treier M.** The
593 murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation
594 and ovary maintenance. *Development (Cambridge, England)* 131: 933-942, 2004.
- 595 48. **St John MA, Tao W, Fei X, Fukumoto R, Carcangiu ML, Brownstein DG, Parlow AF,**
596 **McGrath J, and Xu T.** Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian
597 tumours and pituitary dysfunction. *Nature Genetics* 21: 182-186, 1999.

- 598 49. **Stocco DM.** StAR protein and the regulation of steroid hormone biosynthesis. *Annual*
599 *Review of Physiology* 63: 193-213, 2001.
- 600 50. **Thompson WE, Powell J, Thomas KH, and Whittaker JA.** Immunolocalization and
601 expression of the steroidogenic acute regulatory protein during the transitional stages of rat
602 follicular differentiation. *J Histochem Cytochem* 47: 769-776, 1999.
- 603 51. **Tran H, Brunet A, Griffith EC, and Greenberg ME.** The many forks in FOXO's road. *Sci*
604 *STKE* 2003: RE5, 2003.
- 605 52. **Treier M, Gleiberman AS, O'Connell SM, Szeto DP, McMahon JA, McMahon AP, and**
606 **Rosenfeld MG.** Multistep signaling requirements for pituitary organogenesis in vivo. *Genes*
607 *Dev* 12: 1691-1704, 1998.
- 608 53. **Trombly DJ, Woodruff TK, and Mayo KE.** Roles for transforming growth factor beta
609 superfamily proteins in early folliculogenesis. *Seminars in Reproductive Medicine* 27: 14-23,
610 2009.
- 611 54. **Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P,**
612 **Fausser BC, and Themmen AP.** Anti-Mullerian hormone expression pattern in the human
613 ovary: potential implications for initial and cyclic follicle recruitment. *Molecular Human*
614 *Reproduction* 10: 77-83, 2004.
- 615 55. **Woodruff TK, D'Agostino J, Schwartz NB, and Mayo KE.** Dynamic changes in inhibin
616 messenger RNAs in rat ovarian follicles during the reproductive cycle. *Science (New York)*
617 239: 1296-1299, 1988.
- 618 56. **Xia H, Qi H, Li Y, Pei J, Barton J, Blackstad M, Xu T, and Tao W.** LATS1 tumor
619 suppressor regulates G2/M transition and apoptosis. *Oncogene* 21: 1233-1241, 2002.
- 620 57. **Xu T, Wang W, Zhang S, Stewart RA, and Yu W.** Identifying tumor suppressors in
621 genetic mosaics: the *Drosophila* *lats* gene encodes a putative protein kinase. *Development*
622 *(Cambridge, England)* 121: 1053-1063, 1995.
- 623 58. **Zeng Q and Hong W.** The emerging role of the hippo pathway in cell contact inhibition,
624 organ size control, and cancer development in mammals. *Cancer Cell* 13: 188-192, 2008.
- 625 59. **Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, Zheng P,**
626 **Ye K, Chinnaiyan A, Halder G, Lai Z-C, and Guan K-L.** Inactivation of YAP
627 oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth
628 control. *Genes Dev* 21: 2747-2761, 2007.
- 629 60. **Zlotogora J, Sagi M, and Cohen T.** The blepharophimosis, ptosis, and epicanthus inversus
630 syndrome: delineation of two types. *American Journal of Human Genetics* 35: 1020-1027,
631 1983.
- 632
633

634 **Figure Legends:**

635 **Figure 1. LATS1 is co-immunoprecipitated with FOXL2.** Mammalian CHO cells were
636 transfected with an empty expression vector (-) or FLAG-FOXL2 expression construct (+).
637 Twenty-four hours after transfection, the cells were lysed, and the lysates were
638 immunoprecipitated with a control mouse IgG or an antibody to FLAG. The lysates (Lysate) and
639 immunoprecipitates (IP) were analyzed by immunoblotting with FOXL2 and LATS1 antibodies.
640 When the empty pFLAG-CMV-2 vector was used as a template, FOXL2 was not synthesized
641 (Lysate, -), but when the pFLAG-CMV-2 -FOXL2 construct was used as a template, FOXL2
642 was synthesized (Lysate, +). Some endogenous LATS1 expression was also detected in the
643 lysates prior to immunoprecipitation. When these lysates were immunoprecipitated with mouse
644 IgG, a faint band was obtained for FLAG-FOXL2 in immunoprecipitates from cells expressing
645 FLAG-FOXL2, and no band was obtained for LATS1 (IP: Mouse IgG). When the lysates were
646 immunoprecipitated with an antibody to FLAG, endogenous LATS1 was co-immunoprecipitated
647 in cells expressing FLAG-FOXL2 (IP: FLAG, +) but not in cells expressing the empty
648 expression vector (IP: FLAG, -).

649
650 **Figure 2. FOXL2 is phosphorylated *in vitro*.** A. Mammalian CHO cells, which do not express
651 endogenous FOXL2, were transfected with the pcDNA3-FOXL2 expression construct (+) or
652 with an empty pcDNA3 expression vector (-). Twenty-four hours after transfection, the cells
653 were lysed and treated with phosphatase inhibitor alone (PI), alkaline phosphatase alone (AP), or
654 alkaline phosphatase and phosphatase inhibitor (AP+PI). The proteins were then analyzed by
655 immunoblotting (IB). Two FOXL2 bands are seen in lysates treated with phosphatase inhibitor
656 (PI), the lower of which corresponds in size to FOXL2 (41). The upper band was eliminated in
657 lysates treated with alkaline phosphatase (AP), but was preserved in lysates treated with both

658 alkaline phosphatase and phosphatase inhibitor (AP+PI). B. CHO cells were transfected with
659 pFLAG-FOXL2 expression construct (+) or an empty expression vector (-). Twenty-four hours
660 after transfection, the cells were lysed and the resulting proteins were immunoprecipitated with
661 FLAG or mouse IgG (control) antibodies, and analyzed by immunoblotting with FOXL2 and
662 phospho-serine antibodies. Two FOXL2 bands were visualized using the FOXL2 antibody in the
663 FLAG immunoprecipitates, but not in the mouse IgG precipitates, the larger of which was also
664 detected by the phospho-serine antibody (pSerine-FOXL2). C. Lysates treated with alkaline
665 phosphatase in (A) were immunoblotted with the FOXL2 and phospho-serine antibodies.
666 A single FOXL2 band was seen in the FLAG immunoprecipitates, but not in the mouse IgG
667 precipitates. No bands were detected using the phospho-serine antibody.

668

669 **Figure 3. FOXL2 is phosphorylated by LATS1.** A. CHO cells were transfected with the
670 pcDNA3-His-Xpress LATS1 construct, lysed, and Xpress-tagged LATS1 was purified using a
671 LATS1 antibody for use as the kinase. De-phosphorylated FLAG-tagged FOXL2 proteins were
672 used as substrates in kinase assays. Different amounts of Xpress-tagged LATS1 were mixed with
673 FLAG-tagged FOXL2 for kinase phosphorylation, and the reaction products were analyzed by
674 immunoblotting with antibodies to FOXL2, LATS1 and phospho-serine. In the absence of
675 Xpress-LATS1, FOXL2 was not phosphorylated at a serine residue. In the presence of increasing
676 concentrations of Xpress-LATS1, the amount of phospho-serine-FOXL2 (pSerine-FOXL2)
677 increased. B. In order to determine whether LATS1 is a kinase for FOXL2, CHO cells were
678 transfected with pcDNA3-His-Xpress LATS1 constructs, either the wild type (Wt Xpress-
679 LATS1) or the kinase inactive D846A mutant (Mt Xpress-LATS1), lysed, and purified using the
680 Xpress antibody for use as kinases. Xpress-tagged wild type LATS1 or Xpress-tagged mutant

681 LATS1 D846A was mixed with de-phosphorylated FLAG-tagged FOXL2 for kinase
682 phosphorylation. A strong phosphoserine-FOXL2 band was observed when wild-type LATS1
683 was used as the kinase, but only a very faint band was observed when the same concentration of
684 the D846A mutant LATS1 was used.

685

686 **Figure 4. LATS1 gene is expressed in developing mouse ovary.** A. Total RNA was extracted
687 from whole ovaries of fetal (17.5 dpc), immature (D13 and D23), and adult (adult) mice, reverse
688 transcribed, PCR amplified with LATS1 primers, and analyzed by electrophoresis. Human Ovary
689 PCR-Ready cDNA (Ambion, Austin, TX) was used as a positive control (+), and water was used
690 as a negative control (-). The identities of the LATS1 PCR products were confirmed by
691 sequencing. B. FOXL2 expression in the same cDNAs (41) is shown for comparison.

692

693 **Figure 5. FOXL2 and LATS1 are co-expressed in mouse granulosa cells.**

694 Immunohistochemical staining was performed on serial sections of ovaries from immature (21
695 day-old) mice, which do not contain mature (steroidogenic) follicles, incubating without primary
696 antibody (as a control) or antibodies to FOXL2 or LATS1, followed by counterstaining with
697 hematoxylin. Both FOXL2 and LATS1 were expressed in granulosa cells of small (arrow) and
698 medium (arrowhead) follicles.

699

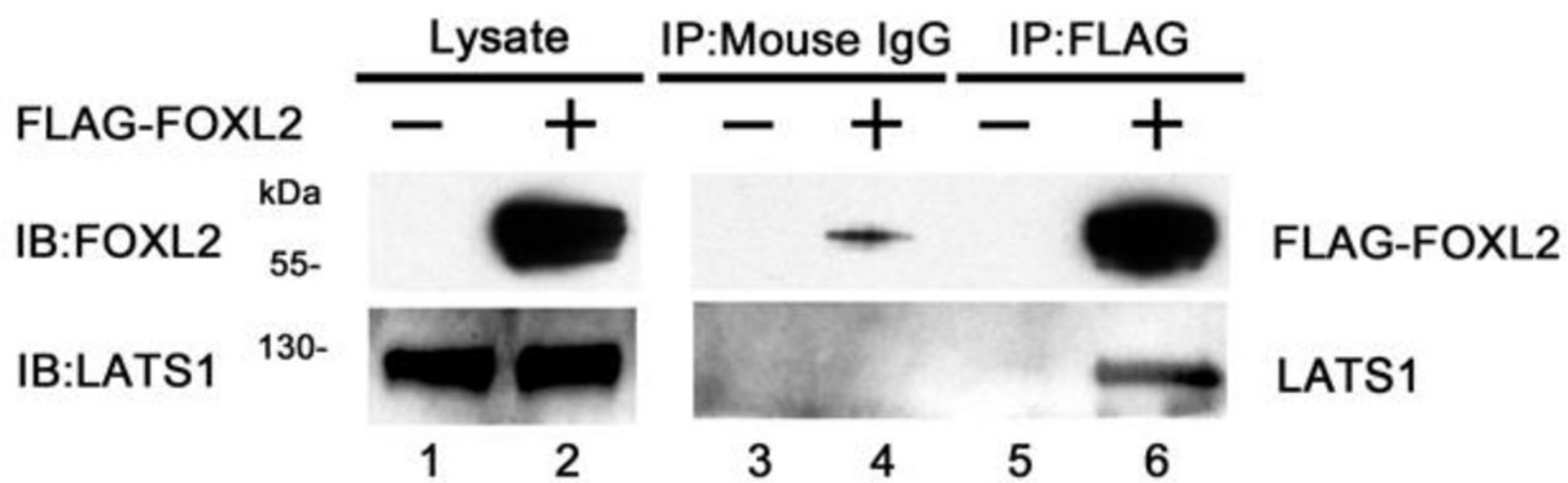
700 **Figure 6. LATS1 enhances the repressive effect of FOXL2 on the StAR promoter.** Wild-
701 type CHO cells and CHO cells expressing FOXL2 (FOXL2 stable cells) were transfected with
702 100ng of the pcDNA3.1-His-Xpress-LATS1 expression construct (Wt LATS1), 100 ng of the
703 kinase-inactive D846A mutant LATS1 expression construct (Mt LATS1), or 100ng of the empty

704 pcDNA3.1-His vector backbone (empty vector) and the -95-bp StAR promoter-luciferase
705 plasmid. The resulting luciferase activities were determined 24 hours after co-transfection.
706 Luciferase activity values were normalized against activity levels in FOXL2 stable cells
707 transfected with the StAR promoter plasmid in the presence of the empty vectors (value = 1).
708 One-way ANOVA revealed that CHO cells were transfected with wild-type or mutant LATS1 in
709 the absence of FOXL2, luciferase activities were similar to the control (a). In the presence of
710 FOXL2 (FOXL2 stable cells (b)) luciferase activity was significantly reduced compared to that
711 in CHO cells without FOXL2 (a) ($p < 0.05$). When FOXL2 stable cells were transfected with
712 wild-type LATS1 (c), the luciferase activity of the StAR promoter was significantly reduced
713 compared to control-transfected stable cells (b) ($p < 0.05$). When FOXL2 stable cells were
714 transfected with the kinase-inactive LATS1 mutant (MtLATS1), luciferase activity was restored
715 to levels in control-transfected stable cells (b).

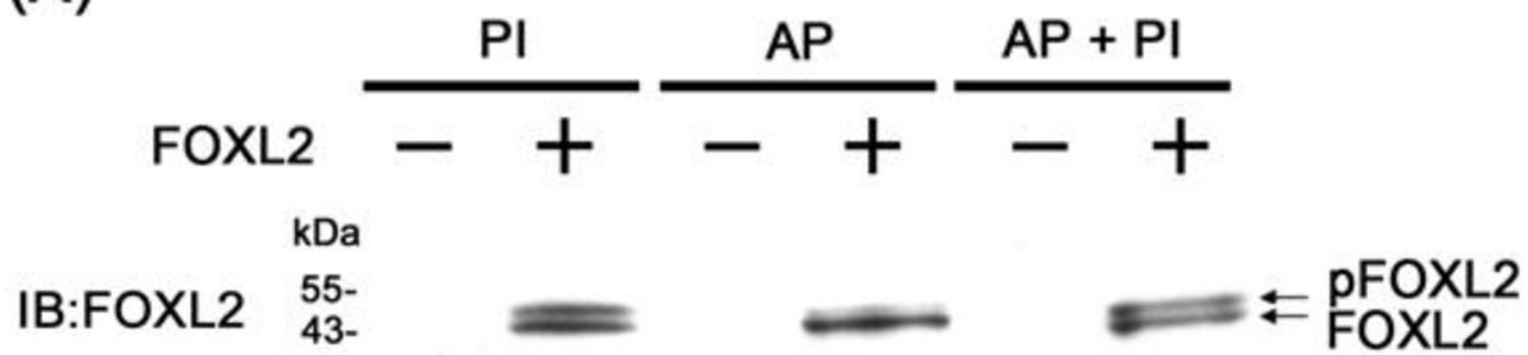
716

717

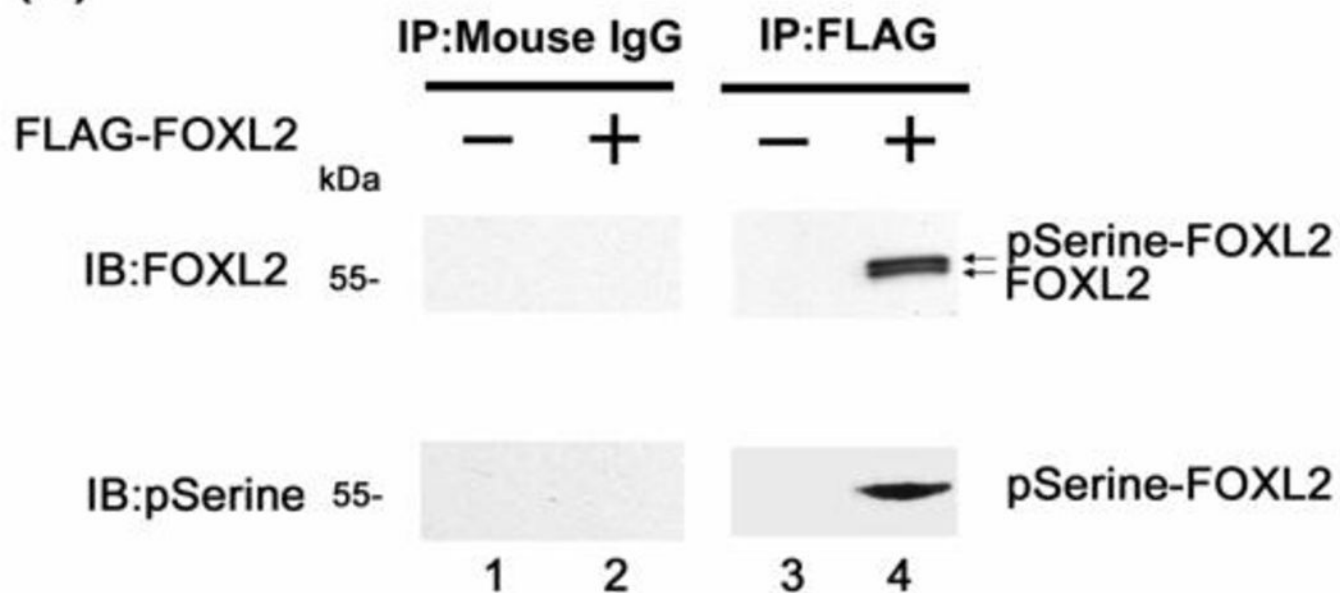
718



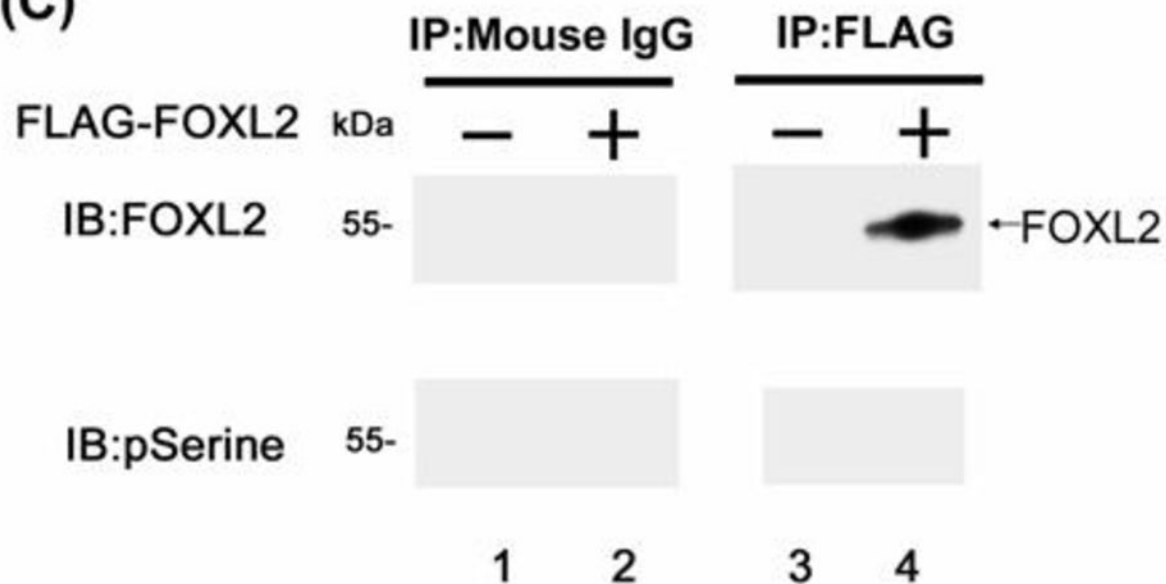
(A)

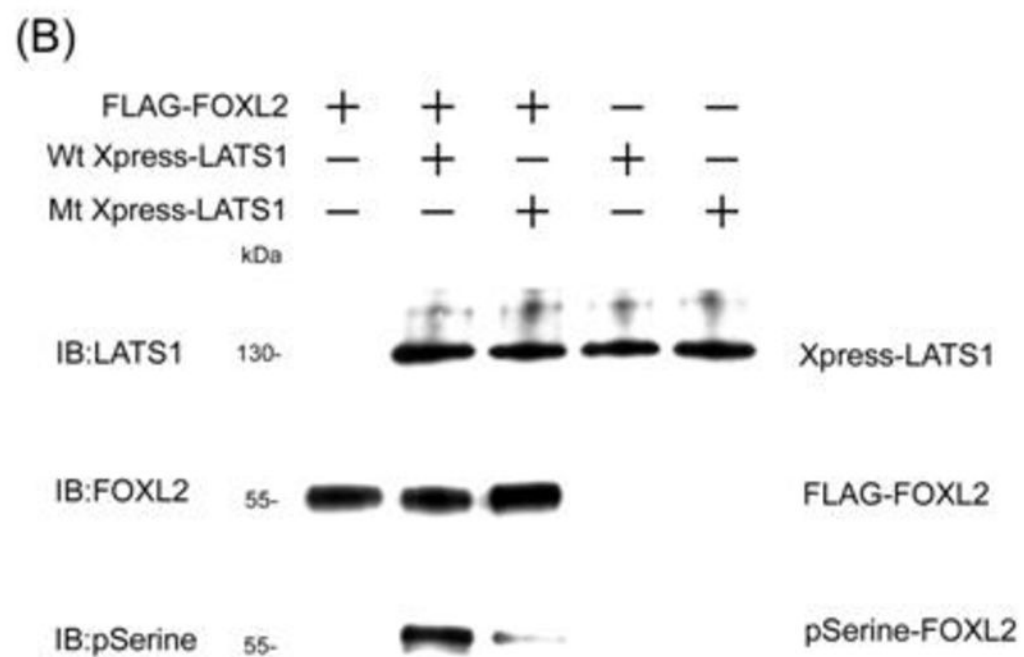
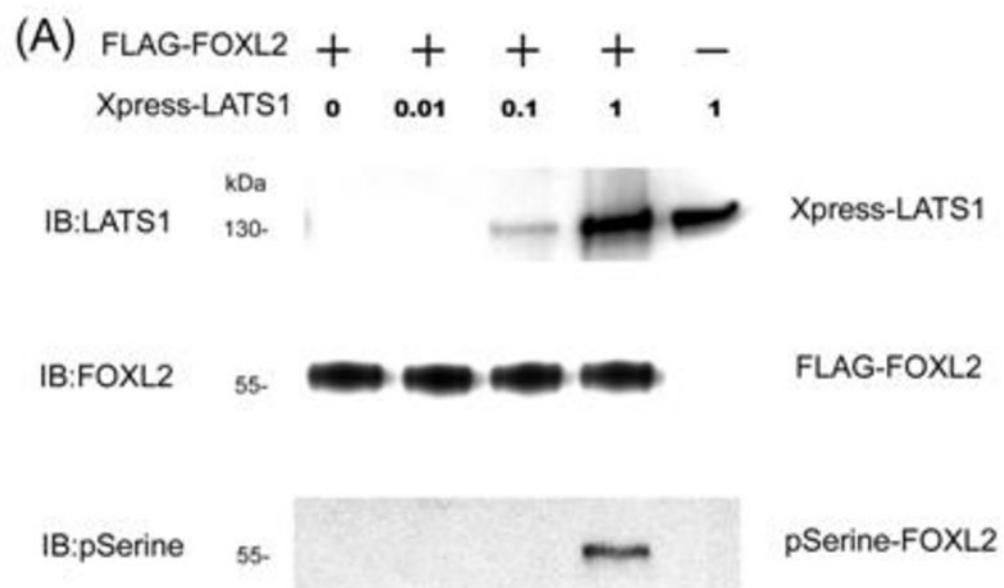


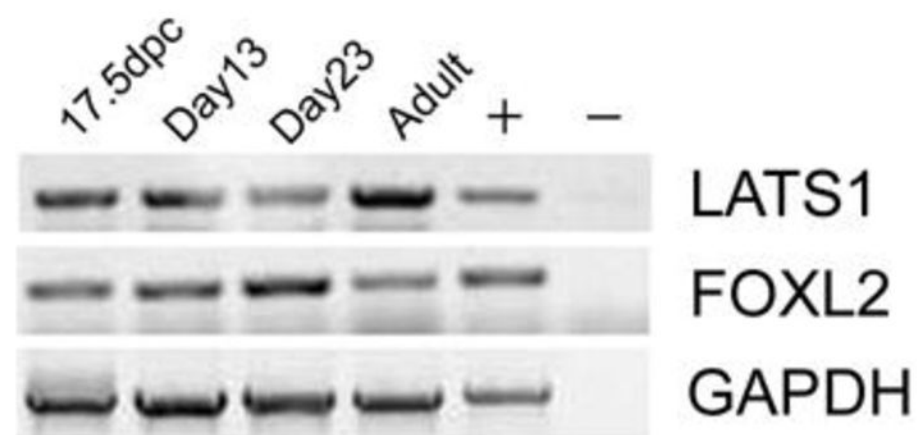
(B)



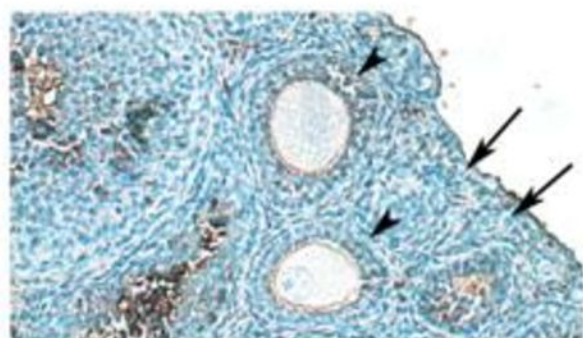
(C)



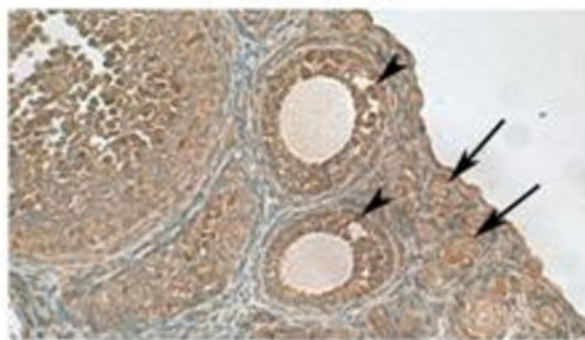




Control



FOXL2



LATS1

