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## At the crossroads of fertility and metabolism

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1       **At the crossroads of fertility and metabolism: the importance of AMPK-dependent**  
2                   **signaling in female infertility associated with hyperandrogenism**

3

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30

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32

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40

## Abstract

41

42

43 **Study question:** What biological processes are linked to the signaling of the energy sensor  
44 AMP-activated protein kinase (AMPK) in mouse and human granulosa cells (GCs)

45 **Summary answer:** The lack of  $\alpha 1$ AMPK in a transformed human granulosa cell line (KGN)  
46 impacted cell cycle, adhesion, lipid metabolism, and steroidogenesis whereas in a transgenic  
47 mouse model, a hyperandrogenic response was accompanied by higher  $3\beta$ HSD and p450scc  
48 levels in the ovaries and increases in antral follicles. The  $\alpha 1$ AMPK gene expression from  
49 primary granulosa cells from non-obese women with PCOS (Polycystic ovary syndrome)  
50 showed a lower expression than those without it.

51 **What is known already:** AMPK is expressed in the ovarian follicle, and its activation by  
52 pharmacological medications such as metformin inhibits the production of steroids and  
53 androgens. PCOS is responsible for infertility in approximately 5–20% of women of  
54 childbearing age. Possible treatments for PCOS include reducing body weight, improving  
55 lifestyle, and the administration of a combination of drugs (e.g., clomiphene citrate with  
56 metformin) to improve insulin resistance.

57 **Study design, size, duration:** AMPK signaling was evaluated by analyzing differential gene  
58 expression in KGN cells with and without silencing  $\alpha 1$ AMPK using CRISPR/Cas9. *In vivo*  
59 studies included the use of a  $\alpha 1$ AMPK knock-out mouse model to evaluate the role of  $\alpha 1$ AMPK  
60 in folliculogenesis and fertility. Expression of  $\alpha 1$ AMPK was evaluated in primary human  
61 granulosa-luteal cells from women with and without PCOS.

62 **Participants/materials, setting, methods:**  $\alpha 1$ AMPK was disrupted in KGN cells and a  
63 transgenic mouse model. Cell viability, proliferation, and metabolism were evaluated.  
64 Androgen production was evaluated by analyzing protein expression of relevant enzymes of

65 steroid pathway by Western blots and compound levels obtained from *in vitro* and *in vivo*  
66 models by mass spectrometry. Differential gene expression in human GC was obtained by  
67 RNAseq. Analysis of *in vivo* folliculogenesis was performed by histology and  
68 immunochemistry, including evaluation of the AMH marker. The  $\alpha$ 1AMPK gene expression  
69 was evaluated by qPCR in primary granulosa cells obtained from non-obese women with (n=8)  
70 and without PCOS (n=9).

71 **Main results and the role of chance:** Silencing of  $\alpha$ 1AMPK in KGN increased cell  
72 proliferation ( $p<0.05$ , n=4), promoted the use of fatty acids over glucose, and induced a  
73 hyperandrogenic response resulting from the upregulation of two of the enzymes involved in  
74 steroid production, namely 3 $\beta$ HSD and p450scc ( $p<0.05$ , n=3). Female mice deficient in  
75  $\alpha$ 1AMPK had a 30% decrease in their ovulation rate ( $p<0.05$ , n=7) and litter size, a  
76 hyperandrogenic response ( $p<0.05$ , n=7) with higher levels of 3 $\beta$ HSD and p450scc levels in  
77 the ovaries, and an increase in the population of antral follicles ( $p<0.01$ , n=10) compared to  
78 controls. Primary granulosa cells from non-obese women with PCOS had lower  $\alpha$ 1AMPK gene  
79 expression than the control group ( $p<0.05$ , n=8-9).

80 **Large scale data:** N/A.

81 **Limitations, reasons for caution:** The human KGN is a not fully differentiated, transformed  
82 cell line. As such, to confirm the role of AMPK in GC and the PCOS phenotype, this model  
83 was compared to two others: an  $\alpha$ 1AMPK transgenic mouse model and primary differentiated  
84 granulosa-lutein cells from non-obese women undergoing IVF (with and without PCOS). A  
85 clear limitation is the small number of PCOS patients utilized in this study and that the  
86 collection of primary GC cells was performed after hormonal stimulation.

87 **Broader implications of the findings:** Our results reveal that AMPK is directly involved in  
88 steroid production in human granulosa cells. In addition, AMPK signaling was associated with  
89 other processes frequently reported as dysfunctional in PCOS models, such as cell adhesion,  
90 lipid metabolism, and inflammation. Silencing of  $\alpha 1$ AMPK in KGN promoted folliculogenesis  
91 with increases in AMH. Evaluating the expression of the  $\alpha 1$ AMPK subunit could be considered  
92 as a marker of interest in infertility cases related to hormonal imbalances and metabolic  
93 disorders including PCOS.

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96 funded by the French National Research Agency (ANR). The authors report no intellectual or  
97 financial conflicts of interest related to this work .

98 **Trial registration number:** N/A.

99 **Keywords:** fertility, granulosa, AMPK; androgens; PCOS

101

## **Introduction**

102 Polycystic ovary syndrome (PCOS) is one of the leading causes of female infertility (5–  
103 20% of women of childbearing age (El Hayek *et al.*, 2016; Joham *et al.*, 2015)). It is  
104 characterized by hyperandrogenism, infertility, anovulation, and/or ovarian cysts, with a broad  
105 phenotypic expression (including hirsutism and acne) (Rotterdam ESHRE/ASRM-Sponsored  
106 PCOS Consensus Workshop Group, 2004). Nearly 40–60% of patients with PCOS are either  
107 overweight or obese (Ehrmann *et al.*, 2006). As such, intervention therapies for PCOS include  
108 reducing the body weight, improving the lifestyle, and administering a combination of drugs  
109 (e.g., clomiphene citrate with metformin) to overcome insulin resistance and attenuate PCOS-  
110 related endocrine and infertility issues (Fulghesu *et al.*, 2012).

111

112 Metformin therapy has been used to realign metabolic and hormonal dysfunctions with  
113 some positive outcomes after IVF and IVF/ICSI interventions in women with PCOS (Witchel  
114 *et al.*, 2019; Abdalla *et al.*, 2020; Wu *et al.*, 2020). Deciphering the mechanisms of metformin's  
115 actions in the ovaries has pointed to a critical kinase activated by metformin called the 5'-AMP-  
116 activated protein kinase (AMPK) (Tosca *et al.*, 2005, 2007b; Hardie *et al.*, 2016; Faure *et al.*,  
117 2018). Recently, decreases in AMPK protein levels in the ovaries were reported in DHEA-  
118 induced PCOS mouse model (Tao *et al.*, 2017) whereas AMPK activators (AICAR, resveratrol,  
119 metformin) improved fertility. Moreover, a close functional link was reported for androgen  
120 production and AMPK activity in the ovaries from a transgenic mouse model deficient in  
121 LKB1, an AMPK activating kinase gene (Tao *et al.*, 2017). Collectively these studies support  
122 the notion of a critical role of AMPK in granulosa cells linked to both metabolism and fertility.

123 AMPK is a serine/threonine heterotrimeric kinase composed of a catalytic  $\alpha$ -subunit and  
124 two regulatory subunits,  $\beta$ , and  $\gamma$ . All three subunits of AMPK are highly conserved in

125 eukaryotic species (Hardie *et al.*, 2003; Ghillebert *et al.*, 2011). This kinase is activated when  
126 energy reserves are depleted to produce more energy to maintain ATP homeostasis. Activation  
127 of AMPK entails the phosphorylation of the  $\alpha$ -subunit at Thr172. All three subunits of AMPK  
128 are expressed in the ovaries (Tosca *et al.*, 2005; Dupont *et al.*, 2012; Tartarin *et al.*, 2012) as  
129 well as in different follicle cell types (granulosa cells, theca cells, and oocytes) and corpora  
130 lutea. The expression and localization of AMPK in the ovary are conserved across species,  
131 ranging from invertebrates to vertebrates (Guévélo *et al.*, 2013), and within vertebrates, from  
132 mammals to birds (Tosca *et al.*, 2006), cows (Tosca *et al.*, 2007a), pigs (Mayes *et al.*, 2007),  
133 rodents (Tosca *et al.*, 2005; Downs *et al.*, 2010) and humans (Pellatt *et al.*, 2011).

134 In a related but separate role, AMPK may control the crosstalk between energy balance  
135 and reproduction, both processes affected in PCOS (Bertoldo *et al.*, 2014). For instance, glucose  
136 metabolism dysregulation can trigger defects in ovulation and lower oocyte quality (Downs and  
137 Mastropolo, 1994; Pesant and Baillargeon, 2011). Few studies explored the role of AMPK in  
138 fertility despite that silencing of oocyte-specific  $\alpha 1$ AMPK leads to a 30% litter size reduction  
139 (Bertoldo *et al.*, 2015). The specific deletion of  $\alpha 1$ AMPK in murine oocytes altered junctional  
140 protein expression and some aspects of mitochondrial physiology (Bertoldo *et al.*, 2015).  
141 Pharmacological activation of AMPK inhibited the production of progesterone and oestradiol  
142 (Tosca *et al.*, 2005, 2007b), and lower steroidogenesis associated with decreased  $3\beta$ -  
143 hydroxysteroid dehydrogenase ( $3\beta$ -HSD) and p450<sub>scc</sub> gene and protein expression (Tosca *et*  
144 *al.*, 2005, 2007b, 2010). However interesting these studies are, they included the use of cultured  
145 granulosa cells obtained from animal models (rodent and bovine species) and the use of AMPK  
146 activators may lack target specificity. As such, no studies have shown a direct role of AMPK  
147 signaling in ovarian granulosa steroidogenesis *in vivo*.

148



149           To bridge this gap in knowledge, this study assessed the role of AMPK steroidogenesis,  
150 cell proliferation, and survival in *in vitro* and *in vivo* models by silencing the main catalytic  
151 subunit of AMPK,  $\alpha$ 1AMPK, in human granulosa cells, and by utilizing a transgenic mouse  
152 model deficient in  $\alpha$ 1AMPK.

153

154

## Material and Methods

155

### **Animals**

157 Adult animals (10 to 16 week-old mice) wild type ( $\alpha 1$ AMPK<sup>+/+</sup>) or global  $\alpha 1$ AMPK knock-out  
158 mice ( $\alpha 1$ AMPK<sup>-/-</sup>) were described in Jorgensen *et al.* (Jørgensen *et al.*, 2004). Briefly, an IRES-  
159  $\beta$ geo cassette (internal ribosomal entry site- $\beta$ -galactosidase and neomycin phosphotransferase  
160 fusion gene) was inserted to delete a part of the catalytic domain of  $\alpha 1$ AMPK subunit from  
161 amino acids 97–157. Mice were maintained under standard conditions of light (12 h light and  
162 12 h darkness) and temperature with *ad libitum* access to food and water. All animal procedures  
163 followed the European legislation for animal experimentation (Directive 86/609/EEC) for  
164 animal experiments and with French legislation on animal research. The procedure using  
165  $\alpha 1$ AMPK-deficient mice was approved by the Directeur Départemental des Services  
166 Vétérinaires of the Préfecture de Police de Paris (agreement n° 75-886) and the Paris Descartes  
167 University ethics committee (agreement n° CEEA34.BV.157.12). The fertility assessment was  
168 performed by crossing three month-old wild-type male mouse with two  $\alpha 1$ AMPK-deficient  
169 female mice at the same age for two weeks in the same cage. When a vaginal plug was detected,  
170 the mated female was moved to another cage, and then the litter size was counted at birth.

171 To recover blood and ovarian tissue, 10 to 16 month-old wild-type female animals were killed  
172 by cervical dislocation. Ovaries were fixed in bouin solution for histological studies or directly  
173 stored at -80°C for molecular biology analysis. Five days after a vaginal plug, a few females  
174 were also euthanized to recover embryos (5-day post-coitum (dpc)).

175

176 To assess ovaries' response to gonadotropins, oocytes were retrieved following superovulation  
177 induction. Mice were injected i.p. with 5 I.U. of equine chorionic gonadotropin (eCG); 46 h

178 later, they were injected with 5 I.U. of human chorionic gonadotropin (hCG, Intervet, Boxmeer,  
179 Holland). At 12 h later, the oocytes were retrieved and counted.

180

### 181 **Human primary granulosa cells**

182 Primary human Granulosa-Lutein cells (GC) were obtained from non-obese women ( BMI  $\leq$   
183 30 kg/m<sup>2</sup>) with PCOS and without PCOS undergoing *in vitro* fertilization protocols (IVF). The  
184 cells were collected from pre-ovulatory follicles during oocyte retrieval. GC were centrifuged  
185 and used for culture , or the pellets were frozen at -80°C to analyze  $\alpha$ AMPK expression (Table  
186 2). The ovarian stimulation procedure was described in detail previously (Roche *et al.*, 2016).  
187 Ethical approval for this study was obtained from the Research Ethics Board of the CHRU  
188 Bretonneau and written informed consent was obtained from all patients. No patients received  
189 any monetary compensation for participating in the study, and all patients gave written informed  
190 consent before using cells for research purposes. The diagnosis of PCOS was established  
191 according to the Rotterdam criteria (oligomenorrhea and/or anovulation, clinical and/or  
192 biochemical signs of hyperandrogenism, and polycystic ovaries on ultrasonography scan, as  
193 well as the exclusion of other aetiologies that mimic the PCOS phenotype) (Rotterdam  
194 ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004; Teede *et al.*, 2018) by  
195 qualified personnel. The non-obese women without PCOS underwent fertility treatments due  
196 to male hormonal imbalances or tubal obstruction.

197

### 198 **Immortalized granulosa cells**

199 Immortalized human granulosa cells (KGN) were kindly provided by Drs. Masatoshi  
200 Nomura and Hajime Nawata, Kyushu University, Japan (Nishi *et al.*, 2001). KGN cells  
201 maintain most of the physiological characteristics of primary granulosa cells, including

202 functional FSH receptors and aromatase expression. KGN cells were cultured in Dulbecco's  
203 minimal essential medium (DMEM)/F12 medium (Sigma, St. Louis MO) supplemented with  
204 10% fetal calf serum (FCS) and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin  
205 (Sigma, l'Isle d'Abeau Chesnes, France) in a 5% CO<sub>2</sub> atmosphere at 37°C. Only early passages  
206 (under 30) were used. The cells were cultured at 1 million cells/T75cm<sup>2</sup> for qPCR or Western  
207 blot or 96-well dishes (5,000 cells/well for immunocytochemistry and 10,000 cells/well for  
208 other assays). The culture medium was changed every 48 h. Silencing of human  $\alpha$ 1AMPK in  
209 KGN cells was performed after infection with lentiviral particles expressing small guide RNA  
210 (sgRNA) guides targeting the *PRKAA1* gene (encoding  $\alpha$ 1AMPK)  
211 (GAATGGTACTCTTTCAGGAT), establishing KO-KGN cells, and a non-targeted guide  
212 (GTAGGCGCGCCGCTCTCTAC) creating the control cells, called Scr-KGN cells. The  
213 sgRNA guides and lentivirus generation were previously described. (Sujobert *et al.*,  
214 2015)(Grenier *et al.*, 2018). Successfully transfected KGN cells were selected by puromycin.  
215 Clones were tested for  $\alpha$ 1AMPK expression by Western blotting and sequencing amplicons  
216 surrounding the target sequence on *PRKAA1* exon 7 (sequencing primers: forward:  
217 ATCACCAGGATCCTTTGGCA; reverse: TGCTTTCCTTACACCTTGGTG).

218 A second mutant KGN cell line was established after infection with lentiviral particles  
219 expressing firefly luciferase reporter gene under the control of cAMP-responsive element  
220 (Cignal Lenti CRE-Luciferase Reporter, S.A. Biosciences, Qiagen, France) named hereafter  
221 KGN-CRE-Luc cells. The cAMP-dependent activity was followed by evaluating the production  
222 of cAMP (Promega Luciferase Assay Kit, Promega, Charbonnieres, France) in KGN-CRE-Luc  
223 cells after 48 h of treatment with an activator (1 mM dbAMP) or inhibitor of AMPK (Compound  
224 C, Sigma, l'Isle d'Abeau Chesnes, France). Values were presented as relative luminescence  
225 unit/µg protein and expressed as mean  $\pm$  SEM.

226

## 227 **Immunohistochemistry on fixed cells or tissue**

228 Cell proliferation was evaluated by BrdU staining. Cells were labelled with 10  $\mu$ M BrdU for  
229 24 h (Sigma, l'Isle d'Abeau Chesnes, France), then fixed in 4% PFA/PBS, and BrdU-positive  
230 cells were visualized under indirect immunofluorescence. For labeling polarized mitochondria,  
231 living cells were incubated 30 min at 37°C with 200 nM MitoTracker Orange CM-  
232 H2TMRos(Invitrogen, Fisher Scientific, Illkirch, France). Afterward, they were fixed, and  
233 nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

234 For all other protocols, fixed cells were incubated with PBS/0.1 M glycine for 15 min at room  
235 temperature, permeabilized with 0.1% Triton X-100 (w/v) in PBS for 15 min, and non-specific  
236 binding sites were blocked in 2% bovine serum albumin (BSA)/PBS for 15 min. Then, cells  
237 were incubated overnight at 4°C with the following primary antibodies against phospho-ERK,  
238 Glut4,  $\beta$ -cadherin (Cell Signaling Technologies, Beverly, MA, U.S.A.), BrdU and phalloidin  
239 (Sigma, l'Isle d'Abeau Chesnes, France), p53, and AMH (Santa Cruz Biotechnology, CA,  
240 U.S.A.). Mouse or rabbit IgG (Sigma-Aldrich, l'Isle d'Abeau Chesnes, France) was used as a  
241 negative control. All primary antibodies were diluted at 1:100 in 1% BSA/PBS. After  
242 incubating with the primary antibody, cells were incubated for 45 min with goat anti-rabbit IgG  
243 Alexa Fluor® 555 or anti-mouse IgG Alexa Fluor® 488 antibodies (Invitrogen, Fisher  
244 Scientific, Illkirch, France diluted at 1:500 in 1% BSA/PBS). Cells were counterstained with  
245 Hoechst 33342 (Sigma-Aldrich, l'Isle d'Abeau Chesnes, France) and examined using standard  
246 immunofluorescence microscopy or a high content screening (H.C.S.) imaging system on the  
247 ArrayScan VTI HCS Reader (Thermo Fisher Scientific—Cellomics, Pittsburgh, PA, U.S.A.).  
248 Analysis was performed on three independent biological replicates, with a minimum of 3,000  
249 cells per well. Analysis of the surface of cells was quantified using a phase-contrast microscope  
250 and image analysis software (ImageJ Fiji software, version 1.52, 2018, NIH, U.S.A.). A  
251 minimum of 100 cells in three different experiments ( $n = 3$ ) were analyzed per genotype.

252 Immunochemistry was performed on embedded ovaries in paraffin sectioned with a 7- $\mu$ m slice  
253 thickness. Deparaffinized sections were hydrated, microwaved for 5 min in an antigen  
254 unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France), and cooled at room  
255 temperature. After two washes with PBS for 5 min each, non-specific background was removed  
256 by incubation in 2% BSA/PBS for 30 min. Sections were incubated overnight at 4°C with PBS  
257 containing primary antibody (1:100) against  $\beta$ -catenin, *N*-cadherin (Cell Signaling  
258 Technologies, Beverly, MA, U.S.A.), and AMH (Santa Cruz Biotechnology, CA, U.S.A.). The  
259 following day, sections were incubated with a second fluorescent antibody cited above.  
260 Measurements from different sections per animal (three animals per genotype) were analyzed  
261 using ImageJ Fiji software.

262

### 263 **Colony formation assay and migration assay**

264 A total of 10,000 cells were plated in 6-well plates, cultured in 10% FCS, DMEM/F12.  
265 Cells were allowed to form colonies up to 1–2 weeks, with media being replaced every 48 h.  
266 Colonies were stained with 0.2% crystal violet in methanol, dried, and the colony surface was  
267 analyzed with ImageJ Fiji software.

268

269 Migration of KGN cells was assessed using a scratch assay. Cells were seeded into 12-  
270 well tissue culture dishes at  $5 \times 10^5$  cells per well. At confluence, a linear scratch was generated  
271 by using a sterile tip. Then, cells were incubated with fresh culture medium 10% FCS for 4, 8,  
272 and 24 h at 37°C with 5% CO<sub>2</sub>. The data were analyzed using ImageJ Fiji software to estimate  
273 the migration distance ( $\mu$ m).

274

## 275 **Transepithelial electrical resistance (TEER)**

276 KGN cells were cultured at confluence in 24 well tissue culture inserts (0.4  $\mu\text{m}$  pore size, WWR,  
277 Fontenay sous Bois, France). TEER was collected with Millicell-ERS apparatus and  
278 MERSTX01 electrodes (Millipore, Fisher Scientific, Strasbourg, France). The culture was  
279 equilibrated at room temperature before the evaluation. Blanks were considered wells with  
280 Hank's buffered salt solution (Invitrogen, Cergy Pontoise, France), and the background was  
281 composed of empty wells. TEER was calculated according the following equation: TEER  
282 ( $\text{Ohm}/\text{cm}^2$ ) =  $(R_{\text{total}} - R_{\text{control}}) \times A^{-1}$ . 'Rtotal' is the resistance recorded, 'Rcontrol' is the  
283 resistance of the background, and 'A' is the surface of the insert (i.e., 0.33  $\text{cm}^2$ ). Four  
284 independent biological replicates and technical triplicates were performed in total.

285

## 286 **Cell apoptosis and reactive oxygen species assay**

287 Apoptosis was determined by evaluating the activity of the cleaved form of caspase-3, a  
288 mediator of programmed cell death, by using the commercial Caspase-Glo™ 3/7 Assay  
289 (Promega, Charbonnières, France). Analysis was performed on six different cultures in  
290 duplicate for each condition.

291 The ROS-Glo™ H<sub>2</sub>O<sub>2</sub> Assay (Promega, Charbonnières, France) was used to evaluate oxidative  
292 stress in cells after 48 h of culture by using a luminescent microplate reader (Luminoskan  
293 Ascent, V.W.R. International, France).

294

## 295 **Metabolites and Steroid Analysis**

296 Glucose uptake was evaluated by using the Glucose Uptake-Glo™ Assay (Promega,  
297 Charbonnières, France). Lactate concentrations in the culture medium and pyruvate and  
298 cholesterol contents in granulosa cells were evaluated by using the following  
299 spectrophotometric assays, respectively (Sigma, l'Isle d'Abeau Chesnes, France; Clinisciences,  
300 Nanterre, France; Biolab, Maizy, France). Analysis was performed on at least three different  
301 cultures in duplicate for each condition. The results of each assay were normalized per  $10^6$  cells.  
302 Steroid (pregnenolone,  $17\alpha$ -hydroxypregnenolone, dehydroepiandrosterone,  $17\alpha$ -  
303 hydroxyprogesterone,  $\Delta 4$  androstenedione, testosterone, and dihydrotestosterone) levels were  
304 determined by LC-MS/MS (mmol/L) as described by Meunier *et al.* (Meunier *et al.*, 2015).

305 The serum AMH level was evaluated on five control and three transgenic mice by using an  
306 enzyme-linked immunoassay (AMH Gen II assay®) and read with a Beckman Coulter®  
307 (France) as already described in mice (Valeri *et al.*, 2020). The values are expressed in pmol/l.

308

### 309 **Mitochondrial oxygen consumption ( $JO_2$ )**

310 Live, cultured KGN cells were resuspended at a density of  $1 \times 10^6$  cells  $\times$  mL<sup>-1</sup>. The  $JO_2$  (pmol  
311  $O_2 \times$  (s  $\times$   $10^6$  cells)<sup>-1</sup>) was recorded at 37°C with a high-resolution Oxygraph-2K respirometer  
312 (Oroboros, Innsbruck, Austria), and data were acquired with DatLab4 software. The cell's basal  
313 respiration rate was determined by measuring the linear velocity of oxygen consumption, which  
314 reflects the cell's aerobic metabolic activity with physiological substrates in the culture medium.  
315 Oligomycin (0.5  $\mu$ g/mL) was then added to determine the respiration uncoupled to ATP  
316 synthesis  $JO_2$  (basal  $JO_2$  minus oligomycin-insensitive  $JO_2$ ). The uncoupled (maximum)  
317 respiratory rate was assessed by stepwise additions of carbonyl cyanide *m*-  
318 chlorophenylhydrazone (FCCP; 1–2  $\mu$ M) up to an optimal concentration that represented the  
319 capacity of the respiratory chain without the regulation of ATP synthase.



320

**321 Western immunoblotting**

322 Whole ovaries, embryos at 5-dpc, oocytes, and granulosa cells were lysed and exposed to three  
323 freeze/thaw cycles in a lysis buffer. After centrifugation, the supernatants' protein concentration  
324 was determined using a colorimetric assay (DC assay kit; Uptima Interchim, Montmuçon,  
325 France). Equal amounts of proteins were loaded onto 12% SDS-PAGE under reducing  
326 conditions then transferred onto nitrocellulose membranes (Schleicher & Schuell, Ecquevilly,  
327 France) by following the protocol described before (Tartarin *et al.*, 2012). After that,  
328 membranes were incubated overnight at 4°C with the following primary antibodies:

329 phospho- $\alpha$ AMPK (Thr172), proliferating cell nuclear antigen (PCNA), phospho-CREB (Ser  
330 133),  $\beta$ -catenin (Cell Signaling Technologies, Beverly, MA, U.S.A.),  $\alpha$ 1AMPK (Upstate  
331 Biotechnology Inc, Lake, Placid, NY, U.S.A.), vinculin (Sigma-Aldrich, l'Isle d'Abeau  
332 Chesnes, France), anti-Müllerian hormone (AMH) and 3 $\beta$ -hydroxysteroid dehydrogenase  
333 (3 $\beta$ HSD) (Santa Cruz, CA, U.S.A.); steroidogenic acute regulatory protein (StAR) and anti-  
334 p450 side-chain cleavage (p450scc) antibodies were generously provided by Dr. Dale  
335 Buchanan Hales (Department of Physiology and Biophysics, University of Illinois at Chicago,  
336 Chicago, IL, U.S.A.). All commercial antibodies were used at 1:1000 dilution in Western  
337 blotting, except 3 $\beta$ HSD antibodies at 1:500 dilution. Signal was detected by enhanced  
338 chemiluminescence (Amersham Pharmacia Biotech, Orsay France), and the band densities  
339 were quantified using ImageJ Fiji software (N.I.H., U.S.A.). The results are expressed as the  
340 intensity signal in arbitrary units after normalization by an internal standard ( $\alpha$ 1AMPK protein  
341 or vinculin) and correspond to the average of three different experiments.

342

**343 High-throughput RNA sequencing**

344 The 20 RNA sequencing libraries were constructed from 1  $\mu$ g of total RNA extracted  
345 from Scr-KGN cells or KO-KGN cells using Illumina TruSeq RNA Sample Prep (Illumina, San  
346 Diego, CA, U.S.A.). Sequencing was performed on an Illumina HiSeq 3000 apparatus using  
347 the Illumina TruSeq Standard mRNA kit v2 to obtain stranded paired-end reads (2 x 150 bp).  
348 The analysis pipeline of sequence data was performed using the local instance of Galaxy  
349 (<https://galaxyproject.org>) at the Toulouse Midi-Pyrénées bioinformatics platform  
350 (<http://sigenae-workbench.toulouse.inra.fr/>) as already described (Talebi *et al.*, 2018). The  
351 FastQ files and metadata were submitted to the European Nucleotide Archive (ENA) at EMBL-  
352 EBI under accession number PRJEB46048  
353 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB46048>).

354 The cleaned paired-end reads were combined and mapped using STAR2 against the  
355 human genome assembly (GRCh38.p13) annotated with the Ensembl *Homo\_sapiens*  
356 GRCh38.104 annotation version. For each time condition (Scr-KGN cells and KO-KGN cells),  
357 the 20 files (n = 10 for Scr-KGN samples and n = 10 for KO-KGN samples) with raw counts  
358 were merged to obtain a final count file for statistical analysis of differential gene expression  
359 under AMPK depletion.

360

### 361 **Differentially Expressed Gene analysis**

362 SARTools R package 1.3.2 (Varet *et al.*, 2016) was used for the descriptive and diagnostic  
363 analyses, keeping all samples. Raw counts were pre-filtered for low counts by keeping genes  
364 with at least 1 count per million across 10 out of 20 samples, and counts were normalized for  
365 library size. A generalized linear model was fitted on the normalized raw count data using the  
366 DESeq2 R package version 1.12.3 (Love *et al.*, 2014) to identify the differentially expressed  
367 genes (DEGs) between control Scr-KGN (n = 10) and KO-KGN (n = 10) cells. The raw P-

368 values were adjusted for multiple testing using the Benjamini-Hochberg procedure to control  
369 for the false discovery rate (FDR) (“Controlling the False Discovery Rate: A Practical and  
370 Powerful Approach to Multiple Testing,” 1995). Genes with an adjusted P-value lower than  
371 0.05 (FDR < 5%) were considered as DEGs and selected for subsequent analyses using  
372 Metascape (<http://metascape.org/>). Metascape is a gene annotation and analysis resource (Zhou  
373 *et al.*, 2019) to perform functional enrichment analysis, including a canonical, KEGG, and  
374 Reactome pathway analysis of the overlapping (DEGs) in all three databases.

375

### 376 **RNA extraction, Reverse transcription, and *Real-time quantitative PCR (qPCR)***

377 Total RNA from granulosa cells was extracted with Trizol reagent (Invitrogen, Cergy Pontoise,  
378 France). Total RNA (1 µg) was denatured and retrotranscribed with Moloney murine leukemia  
379 virus (MMLV) reverse transcriptase, 15 U (Promega, Charbonnières, France) in a 20 µl reaction  
380 mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 200 µM dNTP  
381 (Amersham, Piscataway, NJ), 50 pmol of oligo(dT)<sub>15</sub> and 5 U of ribonuclease inhibitor at 37°C  
382 for one h. Targeted cDNAs were quantified by real-time PCR using SYBR Green Supermix  
383 (Bio-Rad, Marnes la Coquette, France) and 250 nM of specific primers (Table 1). Each reaction  
384 was performed in duplicate and included a negative control (without template DNA). The cycle  
385 threshold (Ct) values were calculated automatically and analyzed using CFX Manager™  
386 Software (version 3.1). The relative cDNA number was calculated using the geometric mean  
387 of the Ct values from the following normalizing genes ACTB, EEF1A1, and GAPDH  
388 (Vandesompele *et al.*, 2002).

389

### 390 **Statistical analysis**

391 All data are presented as means  $\pm$  SEM. Unpaired Student's t-test was used to compare the two  
392 genotypes (KO-KGN cells vs. Scr-KGN cells or WT mice vs. KO mice). In the case of variance  
393 heterogeneity, the Mann and Whitney U test was used to compare means between two groups.  
394 Values were determined to be significant when  $p \leq 0.05$  ; \*,  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  
395  $p < 0.001$ .

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## Results

399

### **1. Confirmation of the silencing of $\alpha$ 1AMPK in human granulosa cells**

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To assess the role of AMPK on granulosa cells, we silenced the main catalytic subunit of AMPK  $\alpha$ 1AMPK in human KGN cells by using the sgRNA system developed by Tamburini(Grenier *et al.*, 2018). Sequencing the *PRKAA1* gene confirmed the presence of a sgRNA-induced mutation in exon 7 (**Figure 1A**). The KO-KGN clone showed negligible  $\alpha$ 1AMPK Thr172 phosphorylation and  $\alpha$ 1AMPK protein levels (**Figure 1B**).

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### **2. *In vitro* silencing $\alpha$ 1AMPK stimulates proliferation, adhesion, and faster migration in KGN cells**

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We first investigated the effects of silencing  $\alpha$ 1AMPK on cell viability and proliferation. While KO-KGN cells did not undergo apoptosis or stabilize tumor protein 53 (*TP53*; **Supplemental Data 1A and 1B**), they showed higher proliferation evidenced by the greater cell colony area (1.4-fold higher;  $p < 0.01$ , **Figure 2A**); higher number of BrdU-positive cells (2-fold;  $p < 0.05$ ) (**Figure 2B**); higher percentage of cells with phosphorylated-ERK (**Figure 2C**); and higher content of PCNA ( $p < 0.05$ ) (**Figure 2D**). The hyperproliferation was associated with a reduction of the size of granulosa cells (**Figure 2E**) and a migration estimated (by the scrap assay) 16% faster at 24 h than that in Scr-KGN cells ( $p < 0.05$ ) (**Figure 2F**). Additionally, the cell-cell adhesion of KO-KGN cells was more robust than that of Scr-KGN cells supported by the increased electrical resistance of the cell layer evaluated by TEER (**Figure 2G**) and the higher  $\beta$ -catenin content observed by immunochemistry and Western blot (**Supplemental Data 1C and 1D**).

### 421 3. Silencing $\alpha$ 1AMPK alters glucose metabolism in KGN

422 It has been reported that hyperproliferation is associated with lower mitochondrial  
423 energy metabolism such as described for cancer cells (*Faubert et al., 2015*). In support of this  
424 concept, hyperproliferative KO-KGN cells had a modest lower polarized mitochondria density  
425 per cell (13% decrease,  $p < 0.05$ ) than Scr-KGN cells (**Figure 3A**) suggesting that silencing  
426 AMPK did influence mitochondrial mass significantly. However, the ATP-linked oxygen  
427 consumption rate under basal conditions in KO-KGN cells was half than that of Scr-KGN cells,  
428 and the uncoupled or maximal rate in KO-KGN cells was 0.7-fold of that of Scr-KGN cells ( $p$   
429  $< 0.05$ ; **Figure 3B**). The lower electron transport capacity (phosphorylating and non-  
430 phosphorylating) was associated with a 40% reduction in the levels of hydrogen peroxide  
431 ( $H_2O_2$ ) and reactive oxygen species (ROS. ( $p < 0.01$ ), as well as a 20% reduction in Cellrox  
432 immunostaining, a fluorescence probe activated by ROS ( $p < 0.05$ ) (**Supplemental Data 2**).  
433 These results indicated that silencing AMPK signaling had a more profound impact on  
434 mitochondrial function (30 to 50%) than on mitochondrial mass (13%).

435 In addition, lower glucose uptake was observed in KO-KGN cells (by 70%;  $p < 0.05$ )  
436 associated with a reduction in the expression of the glucose transporter insulin-dependent  
437 GLUT4 (by 30%;  $p < 0.01$ ) (**Figure 3C and 3D**). Despite the lower glucose uptake, activation  
438 of glycolysis is inferred by the substantial increase in lactate in KO-KGN cells compared to  
439 controls (1.8-fold). The higher pyruvate levels in KO-KGN cells compared to Scr-KGN cells  
440 is suggestive of lower activity of the pyruvate dehydrogenase complex (**Figures 3E and 3F**),  
441 probably as a result of higher PDH kinase activity and/or lower mitochondrial function. Indeed,  
442 *in vivo* studies suggested that AMPK regulates pyruvate entry into the mitochondrion (*Klein et*  
443 *al., 2007; Buresova et al., 2020*).

444

#### 445 **4. Silencing AMPK stimulates steroidogenesis in KGN cells**

446 The primary function of granulosa cells is the production of steroids, which evolve  
447 during follicular maturation. The cholesterol content, precursor molecule of steroid synthesis,  
448 was 30% higher in KO-KGN cells (**Figure 4A**). This higher cholesterol content seemed to  
449 facilitate androgen production (6-fold) supported by the higher levels of Delta4  
450 androstenedione (D4), testosterone (TS), and dihydrotestosterone (DHT) ( $p < 0.01$ ) (**Figure**  
451 **4B**). This hyperandrogenism was associated with higher expression of two enzymes involved  
452 in steroid production (p450scc and 3 $\beta$ HSD), 1.2- and 1.7-fold, respectively, in KO-KGN cells  
453 compared to Scr-KGN cells ( $p < 0.05$ ) (**Figure 4C and 4D**), without changes in the expression  
454 of the cholesterol carrier StAR. Taken together these results show that silencing AMPK  
455 increases steroid enzymes' contents. This is also supported by the 3-fold increase in a cAMP-  
456 dependent factor, the pSer-133 CREB transcription factor ( $p < 0.01$ ) (**Figure 4C and 4D**), and  
457 the induction of the reporter gene (luciferase) driven by the cAMP response element (the  
458 response element for CREB) under both basal and dbAMP-stimulated conditions ( $p < 0.05$ )  
459 (**Figure 4E**) when AMPK was inhibited by Compound C.

460

#### 461 **5. Silencing AMPK alters the transcriptome of KGN cells**

462 In order to get a global view of the consequences of the absence of AMPK, we  
463 performed RNA sequencing. RNAseq analysis has revealed 1237 DEG (differentially  
464 expressed genes) (541 DEG down regulated in KO group , 696 DEG up regulated in KO group)  
465 with a  $P < 0.05$ . Genes are involved in the cytoskeleton, extracellular matrix, cell cycle, cell  
466 proliferation, cell locomotion, monocarboxylic acid metabolism, lipid metabolism,  
467 steroidogenesis, and inflammatory response (**Figure 5A** ; volcano-plot, see **Supplemental**  
468 **Data 3**). The results were confirmed and validated by following the gene expression of six

469 candidate genes (interleukin-6, superoxide dismutase 2, Pentraxin 3, collagen type IV alpha 6  
470 chain, matrix metalloproteinase 11, and creatine kinase; **Supplemental Data 4**). Biological  
471 processes enriched in the KO-KGN cells showed the interaction between cytoskeleton  
472 reorganization, metabolism, lipid processes, and immunity processes (**Figure 5B**). Relevant  
473 genes associated with lipid and steroid production were ELOVL7, HSD11B1, CPT1A, and  
474 perilipin 2, including androgen-induced genes, such as vanin 1, genes related to the extracellular  
475 matrix (COL4A5 and COL4A6) and pro-inflammatory genes (IL-6, IL-1B, CCL20, and  
476 CXCL1). Interestingly, a total of 42 down-regulated genes and 14 up-regulated genes in KO-  
477 KGN cells have already been reported in a PCOS database developed from the literature  
478 (PCOSbase) (**Supplemental Data 5**). Gene expression have been confirmed in human primary  
479 granulosa-lutein cells exposed to AMPK inhibitor and also rescued after AMPK re-expression  
480 (**Supplemental Data 6-8**).

## 481 **6. Fertility of $\alpha 1$ AMPK<sup>-/-</sup> female mice is reduced**

482 To place the effects of AMPK in a physiological context, we investigated the fertility of  
483 female  $\alpha 1$ AMPK-deficient mice (**Figure 6A**). The number of live pups per litter was decreased  
484 by 30% in  $\alpha 1$ AMPK<sup>-/-</sup> female mice in comparison to controls ( $p < 0.05$ ) (**Figure 6B**), and  
485 associated with a similar decrease in the number of corpora lutea (**Figure 6C**). To evaluate the  
486 ovary's response independently of the hormones of the hypothalamic-pituitary system, oocytes  
487 were collected and counted after superovulation. The oocyte number was lower in  $\alpha 1$ AMPK<sup>-/-</sup>  
488 mice ( $21.4 \pm 2.4$  oocytes) than in control mice ( $34.2 \pm 1.7$  oocytes;  $p < 0.05$ ) (**Figure 6D**).  
489 Histology of ovaries in  $\alpha 1$ AMPK<sup>-/-</sup> mice has shown presence of all type of follicles but more  
490 secondary follicles formed a ring around the ovary (**Figure 6E**). Analysis of estrous cycle in  
491 transgenic mice revealed a delayed between 2 cycles without change in estrous length (**Figure**  
492 **6F-G**)



493           The drop in the ovulation rate allowed us to further evaluate folliculogenesis. Analysis  
494 of primary and secondary antral follicles in  $\alpha 1$ AMPK<sup>-/-</sup> female mice was increased by 1.3- and  
495 1.5-fold, respectively ( $p < 0.01$ ) (**Figure 7A**). Analysis of atretic follicles by visualization of  
496 pycnotic bodies in follicles revealed only an increase in antral follicles (200-300 $\mu$ m), but not in  
497 primary follicles (50-199 $\mu$ m) or pre-ovulatory follicles (**Figure 7A and Supplemental Data**  
498 **9**). This result was confirmed by a high proportion of AMH-positive follicles, a marker of large  
499 preantral and small antral follicles, in  $\alpha 1$ AMPK<sup>-/-</sup> ovarian tissue ( $p = 0.05$ ) (**Figure 7B and**  
500 **7C**), and elevated AMH levels in the blood; however, the number of pre-ovulatory follicles was  
501 limited in  $\alpha 1$ AMPK<sup>-/-</sup> mice (**Figure 7A**). Expression of  $\beta$ -catenin, which is involved in tissue  
502 architecture, was decreased in secondary antral follicles from  $\alpha 1$ AMPK<sup>-/-</sup> female mice. This  
503 was evidenced by changes in the shape and structure of granulosa cells layers, which were not  
504 cuboidal as in control mice and had a non-linear distribution (**Figure 7D**).

505           Consistent with our results with KO-KGN cells, higher levels of steroids and androgens  
506 were observed in  $\alpha 1$ AMPK<sup>-/-</sup> ovarian tissue and blood compared to those in controls, including  
507 testosterone, pregnenolone, 17 $\alpha$ -hydroxyprogesterone, and  $\Delta 4$ -androstenedione (**Figure 8A-**  
508 **F**). The expression of enzymes involved in steroid production (p450scc and 3 $\beta$ HSD) was also  
509 higher in  $\alpha 1$ AMPK<sup>-/-</sup> ovarian tissue (**Figure 9A-C**). These results confirmed the validity of  
510 using the KGN based on the overlap of outcomes observed with the  $\alpha 1$ AMPK<sup>-/-</sup> mice.

## 511           **7. $\alpha 1$ AMPK content is lower in primary human granulosa cells from non-** 512           **obese women with PCOS**

513           To compare the results obtained *in vitro* with those *in vivo*, we evaluated the gene  
514 expression of  $\alpha 1$ AMPK and  $\alpha 2$ AMPK in primary human granulosa cells retrieved from non-  
515 obese women with and without PCOS, both obtained through IVF protocols. As shown in  
516 **Figure 10A**, a significant 63% reduction in  $\alpha 1$ AMPK mRNA expression was observed in

517 PCOS granulosa cells in comparison to control cells ( $p < 0.05$ ), with no statistical differences  
518 in the gene expression of  $\alpha 2$ AMPK. The  $\alpha 1$ AMPK protein content was slightly but significantly  
519 lower in PCOS granulosa cells (by ~25%) (**Figure 10B**).

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## Discussion

523

524 Our work illustrates the importance of  $\alpha$ 1AMPK for granulosa cell functions and the  
525 potential link with the development of the PCOS phenotype. *In vitro* inactivation of  $\alpha$ 1AMPK  
526 in human granulosa cells leads to changes in the gene expression of proteins involved with the  
527 cytoskeleton, extracellular matrix, cell cycle, immune system, monocarboxylic acids, and lipid  
528 metabolism. This differential expression may explain the issues observed with cell maturation,  
529 glucose metabolism, inflammation and steroid production (**Figure 11**). This phenotype  
530 obtained with a disrupted AMPK axis overlaps with those described in women with PCOS (e.g.,  
531 formation of cysts in the ovary, hyperandrogenism, and insulin resistance (Sirmans and Pate,  
532 2013)).

533 To decipher more precisely the role of AMPK in granulosa cells function, we chose to  
534 limit the use of pharmacological interventions which despite their inhibitory effect on AMPK  
535 may have unintended effects on other kinases. Instead, we developed a strategy to mutate  
536  $\alpha$ 1AMPK gene in human granulosa cells that conserved some properties as the primary cells  
537 such as FSH-sensitivity, steroid production, and expression of AMH. These cells have the  
538 disadvantage of being transformed linked to other gene mutations. Primary granulosa cells from  
539 transgenic mice or human granulosa-lutein cells might also have been available for our study  
540 but they present some disadvantages as well. Human granulosa-lutein cells are retrieved after  
541 hormonal treatment and are differentiated cells with high steroids production and low  
542 proliferation rates; the mouse primary granulosa cells proliferate slowly; and cells preparation  
543 consistency across mice is an issue as well as obtaining follicles at the same stage of  
544 differentiation at the moment of retrieval. The advantage of primary cells used here overcomes  
545 the disadvantage of using heterogenous cell populations associated with lower efficiency in

546 gene manipulation resulting in a mosaic population which may mask the actual effect of the  
547 silencing. We confirmed the results obtained with KGN by using a pharmacological inhibitor  
548 drug (compound C or SBI-0206965) on primary GC in culture (**supplemental data 6-8**) and *in*  
549 *vivo* with ovaries from transgenic  $\alpha 1$ AMPK<sup>-/-</sup> mice, confirming the disruption of steroid  
550 production and adhesion or migration of granulosa cells.

551 Transcriptomics and biological approaches on KGN cells showed the impact on three  
552 main functions: the extracellular matrix and cell proliferation, energy metabolism which has  
553 repercussions on steroid production, and immune function. This last aspect, not explored here,  
554 is worthy of more studies in the future. RNAseq analysis showed dysregulation of genes related  
555 to cytoskeleton reorganization (collagen type IV, matrix metalloproteinase 11, CD9 molecule,  
556 laminin A/C, destrin, an actin-depolymerizing factor). Laminins and collagen proteins are the  
557 most abundant extracellular matrix components of the basal lamina and granulosa cell layers of  
558 ovarian follicles (Rodgers *et al.*, 2001). Modifying the cytoskeleton in ovine granulosa cells  
559 using pharmacological drugs, induced cell rounding, inhibition of proliferation and  
560 progesterone secretion. These observations may support the hypothesis that the cytoskeleton  
561 and extracellular matrix play an essential role in granulosa cell luteinization (Le Bellego *et al.*,  
562 2005). Supporting this notion, several studies have shown that the rigidity of the extracellular  
563 matrix has negative consequences on follicular activation and its growth (West *et al.*, 2007) and  
564 the capacity of migration and adhesion of the cells are critical for the formation of the corpus  
565 luteum ((Rolaki *et al.*, 2007) (Franz *et al.*, 2013). In a mouse model of PCOS, granulosa cells  
566 have been noted to be arranged loosely in fewer (only 1–3) layers (Tao *et al.*, 2017), as we have  
567 observed in our transgenic mouse model. In cultured bovine granulosa cells, among 30  
568 differentially regulated genes related to cell-cell communication were identified by the IPA  
569 tool, with genes affected by AMPK signaling when density changes occurred (Baufeld *et al.*,  
570 2017). Also, in our condition, it is possible that the absence of  $\alpha 1$ AMPK changed the

571 extracellular matrix and induced signals to stimulate granulosa cell proliferation by stimulating  
572 follicular activation as attested by the higher population of pre-antral follicles in  $\alpha 1$ AMPK<sup>-/-</sup>  
573 mice. Furthermore, a study using an AMPK inhibitor described that incubation of primordial  
574 follicles *in vitro* with Compound C increased the growth of the follicle (Jiang *et al.*, 2016). We  
575 can compare these data to a recent study in an ovine model that reported a disruption in the  
576 extracellular matrix structure in the ovaries induced by a maternal environment rich in  
577 androgens and culminated in the acceleration of follicular growth (Monniaux *et al.*, 2020).  
578 Similarly, in our  $\alpha 1$ AMPK<sup>-/-</sup> mice, a high number of large preantral and small antral follicles  
579 were observed with a higher population of AMH-positive follicles in the ovaries. AMH is a  
580 well-established specific marker of this subpopulation of follicles, which is also elevated in  
581 women with PCOS (Visser *et al.*, 2006; Torres-Rovira *et al.*, 2014) and in PCOS-induced  
582 mouse models. Consistent with this view, polycystic ovaries present some abnormalities in the  
583 dynamics of follicles and contain higher numbers of primary and secondary follicles (Franks *et*  
584 *al.*, 2008). AMH levels and ovary follicular development could be improved by administering  
585 an AMPK agonist, metformin, associated with resveratrol or pioglitazone treatments  
586 (Stracquadiano *et al.*, 2018).

587 As expected, energy metabolism was affected in  $\alpha 1$ AMPK<sup>-/-</sup> models (mice and of KO-  
588 KGN cells). We reported, in KO-KGN cells, a decrease in glucose uptake, and a lower content  
589 of the insulin-regulated glucose transporter GLUT4; and lower mitochondrial metabolism  
590 associated with elevated lactate and pyruvate levels. It is now widely recognized that insulin  
591 resistance is present in obese or overweight women and often in lean women with PCOS. The  
592 data obtained from KO-KGN cells are consistent with other studies showing that the levels of  
593 GLUT1 and GLUT3 are lower in PCOS patient-derived iPSCs (Min *et al.*, 2018), and a lower  
594 mitochondria content in blood cells of patients with PCOS, which is inversely correlated to the  
595 level of insulin resistance (Lee *et al.*, 2011). It is known that metabolic disorders are clinically

596 associated with declining fertility (Estienne *et al.*, 2019). In the present study, we showed a  
597 change in the expression of glucose and lipid metabolism enzymes, such as pyruvate  
598 dehydrogenase kinase 4 (entry of pyruvate to Krebs'c cycle) and transaldolase 1 (pentose  
599 phosphate shunt) by the RNAseq analysis of  $\alpha$ 1AMPK-deficient human granulosa cells. PDK4  
600 is known to be induced by AMPK (Houten *et al.*, 2009) and by phosphorylating PDK, inhibits  
601 its activity explaining the higher lactate and pyruvate concentrations in KO KGN cells.  
602 Transaldolase 1 is a key enzyme of the pentose phosphate pathway that generates NADPH for  
603 DNA and RNA syntheses related to proliferation and growth. Others genes of metabolism such  
604 as vanin 1(VNN1), which are regulated by androgens, should be indirectly modified by  
605 hyperandrogenism.

606 Interestingly, the most up-regulated gene was ELOVL fatty acid elongase 7 (ELOVL7)  
607 gene, which catalyzes the formation of neutral lipids and cholesterol esters as a source of *de*  
608 *novo* steroid synthesis. ELOVL7 is also regulated by the androgen pathway through SREBP1  
609 (Tamura *et al.*, 2009). We have confirmed the high cholesterol content in granulosa cells and  
610 the overall consequence on steroid production both *in vitro* and *in vivo* in  $\alpha$ 1AMPK<sup>-/-</sup> transgenic  
611 mice. We also noticed a direct consequence on the hyperactivation of the transcription factor  
612 CREB and enzyme involved in steroid production. Several studies using pharmacological  
613 approaches on granulosa cells described an inhibition of the production of progesterone and  
614 oestradiol in rodent and bovine granulosa cells by the two AMPK activators: AICAR or  
615 metformin (Bowdridge *et al.*, 2015). Metformin is also used as a treatment for patients with  
616 PCOS, restoring ovulation by inhibiting androgen synthesis (Palomba *et al.*, 2005). This  
617 inhibition was partly explained by a decrease in the expression of key enzymes, such as 3 $\beta$ HSD,  
618 p450<sub>scc</sub>, and StAR (Tosca *et al.*, 2005), leading to a reduction of androgen synthesis by thecal  
619 cells (Attia *et al.*, 2001; Mansfield *et al.*, 2003). Quite recently, the secretion of progesterone

620 by human mural granulosa cells was shown to be resistant to AMPK inhibition (Bowdridge *et*  
621 *al.*, 2017) .

622 To investigate the "*in vivo*" possibility of a potential genotype-phenotype correlation,  
623 we examined the fertility of transgenic  $\alpha 1$ AMPK-deficient mice because the phenotype present  
624 several PCOS traits, and we have determined the expression of the  $\alpha 1$ AMPK gene in purified  
625 granulosa cells from patients carrying the PCOS phenotype. Indeed, the  $\alpha 1$ AMPK<sup>-/-</sup> transgenic  
626 mouse model showed irregular cycles, ovulatory dysfunction, dysregulation in folliculogenesis  
627 associated to elevated AMH level and excess in androgen production by the ovaries.  
628 Interestingly, this phenotype is quite similar to those described in rodent model of PCOS  
629 induced by DHEA, where the authors still observed phospho- $\alpha$ AMPK, but a lower total  
630  $\alpha$ AMPK concentrations in the ovaries in comparison to control mice (Tao *et al.*, 2017). Another  
631 recent work found a reduction in AMPK immunoreactivity in follicles from a group of DHEA-  
632 induced PCOS rats compared to a control group (Furat Rencher *et al.*, 2018). The expression  
633 of  $\alpha$ AMPK could be up-regulated by treatment with metformin and resveratrol and was  
634 associated with an improvement of the PCOS phenotype in rat (Furat Rencher *et al.*, 2018).  
635 These results suggest that a lower expression of  $\alpha 1$ AMPK could participate or be associated to  
636 the etiology of PCOS. However, despite the human PCOS syndrome is associated to  
637 multifactorial factors, several PCOS rodent models have been developed through different  
638 strategies and tended to mimic PCOS traits: (i) hyperandrogenism, induced by androgens  
639 (DHEA, DHT) injection or using an aromatase inhibitor, which is one of the most important  
640 features to induce a PCOS phenotype (Caldwell *et al.*, 2014; Abbott *et al.*, 2019; Rodriguez  
641 Paris *et al.*, 2021). More recently, high AMH levels during embryonic stage have shown to  
642 induce a PCOS phenotype during adulthood. Indeed, elevated AMH resulted in maternal  
643 neuroendocrine-driven testosterone excess and masculinized the female fetus (Tata *et al.*, 2018)  
644 ; (ii) by changing diet or stressful environmental conditions ; and (iii) by gene mutation as in

645 transgenic animal models over-expressing LH or nerve growth factor, or presenting a deletion  
646 of PTEN or the plasminogen activator inhibitor-1 (PAI-1) in follicle (Ryu *et al.*, 2019).

647 In our model, we can hypothesize that deficiency in  $\alpha 1$ AMPK in female mice increases  
648 androgen levels through a higher expression of enzymes involved in steroid production as  
649 observed in DHEA or DHT-induced PCOS mouse models and higher AMH levels during  
650 pregnancy. These data provide further evidence for the interest to use AMPK activators  
651 (adiponectin agonist/inducer, metformin...) in a PCOS treatment or to develop a personalized  
652 medicine according to the activity of AMPK in granulosa cells in order to determine the  
653 sensitivity of the patient to the treatment and to adapt it. Furthermore, this model is also of  
654 interest to study the AMPK pathway and the use of AMPK activators in the etiology of  
655 granulosa cancer or development of therapy. It will be interesting to use a strategy to  
656 overexpressed AMPK in ovarian follicle of a PCOS mouse model, we can evaluate the  
657 importance of the AMPK signalling in the treatment of PCOS trait. Moreover, to perform an  
658 inducible deletion of  $\alpha 1$ AMPK specifically in granulosa cells could help us to decipher the  
659 consequence on folliculogenesis if AMPK is absent at early stage (embryo stage) or during  
660 adulthood.

661

662 In summary, granulosa cells from patients with PCOS have lower gene expression of  
663  $\alpha 1$ AMPK. Similarly, a disruption of the  $\alpha 1$ AMPK gene in both *in vitro* human granulosa cells  
664 and *in vivo* in transgenic mice results in a PCOS-like phenotype with irregular cycles, changes  
665 in follicular dynamics, and hyperandrogenism. These changes in folliculogenesis triggers a  
666 reduction in ovulation rate as observed in patients with PCOS or in PCOS-induced mice (Tao  
667 *et al.*, 2017). Our study suggests the involvement of AMPK in granulosa cell functions, and a  
668 dysregulation of AMPK could be implied in the aetiology of the PCOS phenotype. Therefore,



669 novel insights into the mechanism of PCOS associated with AMPK have to be identified. The  
670 development of specific therapeutic targets of AMPK could be of interest to improve the  
671 subfertility associated with a metabolic syndrome such as PCOS.

673

674

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**Figure legends**

687

688 **Table 1. Primers for quantitative Real-Time PCR**689 **Table 2. Biological parameters of patients from control and PCOS groups.**

690 **Figure 1. Mutation of the  $\alpha$ 1AMPK (A)** Sequence of part of exon 7 of the PRKAA1 gene  
691 from KGN genomic DNA (location : 40,765,238 to 40,764,752). **(B)** Western blot analysis of  
692 phospho-AMPK-alpha (Thr172) and  $\alpha$ 1AMPK in KO-KGN (mutant cells) and Scr-KGN  
693 granulosa cells (control cells). Vinculin was used as control. Results are representative of 3  
694 independent experiments.

695

696 **Figure 2. In vitro proliferation ability of KO-KGN cells (A)** Comparison of the KO-KGN  
697 and Scr-KGN granulosa cells colony formation, cells were plated at a very low density ( $1.5 \times$   
698  $10^3$  cells/well) and 2 weeks later, the surface occupied by foci were quantified (n = 4  
699 independent experiments). Representative fields are presented below. **(B)** KGN were cultured  
700 in presence of 10  $\mu$ M BrdU for 24h. The percentage of BrdU positive cells was analyzed by  
701 High Content Screening. Results are representative of 6 independent experiments in triplicate  
702 with at least 3000 cells counted per well. **(C)** Percentage of phosphorylated ERK positive cells  
703 analyzed by High Content Screening (n=3 independent experiments). **(D)** Western blot analysis  
704 of PCNA in KO-KGN and Scr-KGN granulosa cells. Vinculin was used as control. Results are  
705 representative of 3 independent experiments. **(E)** Representative microscopic fields of KO-  
706 KGN and Scr-KGN cell surface. Scale bar = 50  $\mu$ m. (right panel), Quantification of cell surface  
707 ( $\mu$ m<sup>2</sup>) by ImageJ software (measured on at least 300 cells from 3 independent cultures). **(F)**  
708 migration assays. KGN cells were scraped from the culture dish and the mean migration  
709 distance of cells was assessed for 24 h. Quantification of the migration distance for KGN cells

710 is shown as ( $\mu\text{m}$ ) ( $n=4$ ). **(G)** KGN cells were cultured at confluence in 24-well tissue culture  
711 inserts ( $0.4 \mu\text{m}$  pore size) The Trans-epithelial electrical resistance (TEER) was analysed and  
712 reported as ( $\text{Ohm}/\text{cm}^2$ ) 4 different cultures were performed Values are the mean  $\pm$  SEM \*P  
713  $<0.05$ , \*\*P $< 0.01$ , \*\*\*P $<0.001$ .

714

715 **Figure 3. Mitochondria activity in KO-KGN cells.** **(A)** The mitochondrial membrane  
716 potential in KGN cells was analyzed by mitotracker dye. The density and intensity of  
717 mitotracker in each cell was quantified by High Content Screening. Results are representative  
718 of 3 independent experiments. **(B)** KGN suspension ( $1 \times 10^6$  cells/ml) was transferred into a  
719 stirrer 2-ml oxygraph vessel where  $JO_2$  was recorded before and after the successive addition  
720 of  $0.5 \mu\text{g}/\text{ml}$  oligomycin and  $1\text{--}2 \mu\text{m}$  carbonyl cyanide *m*-chlorophenylhydrazone. Data are  
721 represented as means  $\pm$  sem of  $n = 4$  different KGN isolations **(C)** Glucose uptake in KGN  
722 granulosa cells was quantified using the Glucose Uptake-Glo Assay after medium change,  
723 insulin ( $100\text{ng}/\text{ml}$ ) and 30 min incubation with 2DG (2-DeoxyGlucose). ( $n = 4$  independent  
724 experiments). **(D)** intensity of Glut4 immunostaining in each cell was quantified by High  
725 Content Screening. Results are representative of 3 independent experiments. **(E)** Pyruvate  
726 intracellular concentration (ng) per  $\mu\text{g}$  protein content ( $n=3$ ) **(F)** Lactate secretion measured in  
727 culture medium conditioned by KO-KGN and Scr-KGN granulosa cells for 48 h (percentage of  
728 control normalized per 200,000 cells/well) ( $n=7$ ). Values are the mean  $\pm$  SEM \*P  $<0.05$ , \*\*P $<$   
729  $0.01$ , \*\*\*P $<0.001$ .

730

731 **Figure 4. Molecular alteration in steroidogenesis in KO-KGN cells.** **(A)** Intracellular  
732 concentrations of cholesterol in KO-KGN and Scr-KGN granulosa cells ( $n=3$ ). **(B)** KGN  
733 granulosa cells were incubated for 48h with dehydroepiandrosterone (DHEA) and the following

734 steroids were quantified by mass spectrometry:  $\Delta$ 4 androstenedione (D4), testosterone (TS),  
735 dihydrotestosterone (DHT) (n=3). **(C)** Western blot analysis and **(D)** quantification of phospho-  
736 CREB, CREB, 3 $\beta$ -HSD, StAR and P450scc expression in KO-KGN and Scr-KGN granulosa  
737 cells, normalized to vinculin (n=3-5). **(E)** Wild-type KGN cells expressing luciferase under the  
738 control of cAMP response element (Cignal Lenti Reporters, Qiagen) (KGN-CRE-Luc) were  
739 stimulated for 48h with dbAMP 1mM and / or with the AMPK inhibitor (10 $\mu$ M Compound C).  
740 Luciferase assay was performed, and promoter activity values are expressed as relative  
741 luminescence units per $\mu$ g of proteins content. (n=3). Values are the mean  $\pm$  S.E.M. \*P <0.05,  
742 \*\*P < 0.01, \*\*\*P <0.001.

743

#### 744 **Figure 5. Gene enrichment analysis in KO-KGN cells.**

745 Biological function processes or networks of related pathways enriched were constructed  
746 using the Metascape software **(A)** Heatmap of the top15 of GO enriched terms colored by *P*-  
747 values. **(B)** Network of biological processes or related pathways enriched in the KO-KGN  
748 cells samples (n=5) which were closely correlated.

749

750 **Figure 6. Fertility of  $\alpha$ 1AMPK<sup>-/-</sup> female mice.** **(A)** Western blot analysis of the  $\alpha$ 1AMPK  
751 subunit in whole ovary from total knock-out animals (KO,  $\alpha$ 1AMPK<sup>-/-</sup>) versus wild-type mice  
752 (WT,  $\alpha$ 1AMPK<sup>+/+</sup>) (n = 4) **(B)** Fertility analysis of  $\alpha$ 1AMPK<sup>+/+</sup> and  $\alpha$ 1AMPK<sup>-/-</sup> females  
753 mated with wild-type males (n = 29(KO), n=43 (WT)). **(C)** Number of corpora lutea per female  
754 from both genotypes (n = 7). **(D)** Number of oocytes recovered following superovulation  
755 treatment in  $\alpha$ 1AMPK<sup>+/+</sup> and  $\alpha$ 1AMPK<sup>-/-</sup> females (n = 7 per genotype). **(E)** Histology of  
756 ovaries of nontransgenic (left) and homozygotes  $\alpha$ 1AMPK transgenic mice (right). Bar. 200  
757  $\mu$ m. **(F)** Estrous cycle pattern in a representative females. P. proestrus; E. estrus; M. metestrus

758 ; D. diestrus **(G)** Average length of estrus cycle and estrus length. Analysis of the AMH serum  
759 level. Values are the mean  $\pm$  SEM \*P <0.05, \*\*\*P<0.001.

760

761 **Figure 7. Decrease in pre-ovulatory follicles population in  $\alpha$ 1AMPK<sup>-/-</sup>.** **(A)** Representative  
762 picture of atretic and healthy follicle. Bar 100  $\mu$ m. And quantification of healthy and atretic  
763 follicles in the following range of follicles : primary follicles (50-199 $\mu$ m). antral follicles  
764 (200 $\mu$ m-300 $\mu$ m) and pre-ovulatory follicles ( $\geq$  300 $\mu$ m) per ovary were counted in  $\alpha$ 1AMPK<sup>+/+</sup>  
765 and  $\alpha$ 1AMPK<sup>-/-</sup> ovary (n=10 mice per genotype). **(B)** Immunostaining and quantification of  
766 AMH in  $\alpha$ 1AMPK<sup>+/+</sup> and  $\alpha$ 1AMPK<sup>-/-</sup> ovary (n=5 mice per genotype). **(C)** AMH protein content  
767 in whole ovary lysates from  $\alpha$ 1AMPK<sup>+/+</sup> and  $\alpha$ 1AMPK<sup>-/-</sup>, normalized to vinculin.  
768 Quantification was shown in the side of the western-blot. (n=4 mice per genotype). **(D)** AMH  
769 serum level in  $\alpha$ 1AMPK transgenic mice **(E)** Immunostaining and quantification of  $\beta$  catenin  
770 and N-cadherin in preantral follicles. Quantification of intensity was performed by ImageJ  
771 software (n=5 mice per genotype). Values are the mean  $\pm$  SEM \*P <0.05, \*\*P<0.01,  
772 \*\*\*P<0.001.

773

774 **Figure 8. Steroid analysis in  $\alpha$ 1AMPK<sup>-/-</sup> female mice.** The following steroids were analysed  
775 in blood and whole ovary extract from  $\alpha$ 1AMPK<sup>+/+</sup> and  $\alpha$ 1AMPK<sup>-/-</sup> adult females, **(A)**  
776 Pregnenolone, **(B)** 17 $\alpha$ -Hydroxypregnenolone, **(C)** Dehydroepiandrosterone, **(D)** 17 $\alpha$ -  
777 Hydroxyprogesterone, **(E)**  $\Delta$ 4 androstenedione , **(F)** testosterone. (n = 7 mice per genotype).  
778 Values are in mmol/L or  $\mu$ mol/ovary extract as a mean  $\pm$  S.E.M. \*P <0.05, \*\*P<0.01.

779

780 **Figure 9. expression of steroidogenic enzymes in  $\alpha$ 1AMPK<sup>-/-</sup> female mice.** **(A)** the  
781 cholesterol transporter, StAR, **(B)** p450<sub>scc</sub>, and **(C)** 3 $\beta$ HSD expression in whole ovary lysates

782 from  $\alpha 1$ AMPK<sup>+/+</sup> and  $\alpha 1$ AMPK<sup>-/-</sup>, normalized to vinculin. Quantification of the expression of  
783 StAR, p450scc, and 3 $\beta$ HSD in both genotypes were shown in the side of the western-blot. (n =  
784 3 mice per genotype) \*,  $P < 0.05$ . Values are the mean  $\pm$  SEM \* $P < 0.05$ , \*\* $P < 0.01$ .

785

786 **Figure 10. Expression of AMPK in primary human granulosa cells.** (A) left panel.  
787 Testosterone serum level in the obese and non-obese patients (BMI  $\leq 30$ ) and (B.M.I.  $\leq 30$ ).  
788 Right panel. mRNA expression of  $\alpha 1$ AMPK and  $\alpha 2$ AMPK by RT-qPCR in human granulosa  
789 cells retrieved from a group of control non-obese patients (BMI  $\leq 30$ ) (n=8 patients) and a group  
790 of PCOS non-obese patients (BMI  $\leq 30$ ) (n=9 patients).

791 **(B)** Western blot analysis of  $\alpha 1$ AMPK in human granulosa cells retrieved from control or  
792 PCOS patients. Vinculin was used as control. Quantification of total  $\alpha 1$ AMPK was shown  
793 below the Western blot. (n = 6 different patient per group). The results are means  $\pm$  SEM \* $P$   
794  $< 0.05$ , \*\* $P < 0.01$ .

795

796 **Figure 11. Consequence of  $\alpha 1$ AMPK deficiency in granulosa cells:** schematic summary of  
797 the consequences on cell metabolism, steroid production and cell proliferation.

798

799 **Supplemental data 1. Apoptosis marker in KO-KGN** (A) Quantification of the caspase-3/7  
800 cleaved activity in KGN (n = 6 independent experiments). (B) P53 positive cells analyzed by  
801 High Content Screening, nutlin 5 $\mu$ M was used as a positive control to stabilize p53 content (C)  
802 Western blot analysis of  $\beta$  catenin in KGN granulosa cells. Results are representative of 3  
803 independent experiments. (D) Immunostaining and quantification of  $\beta$  catenin in KGN cells.  
804 Quantification of intensity was performed by ImageJ software. Results are representative of 3

805 independent experiments with at least 100 cells counted per experiment. Values are the mean  $\pm$   
806 SEM.

807

808 **Supplemental data 2. Oxidative stress in KO-KGN.** (A) ROS content in KO-KGN and Scr-  
809 KGN cells (n = 4 independent experiments). (B) The intensity of the oxidative stress staining  
810 (Cellrox dye) in each cell was quantified by High Content Screening. Representative fields of  
811 Cellrox staining and a DAPI staining are represented below. Results are representative of 3  
812 independent experiments.

813

814 **Supplemental Figure 3.** volcano plot of  $-\log_{10}$  representing the RNA seq data

815

816 **Supplemental data 4. RT-PCR for significant DEGs**

817 Bar graphs show the results of a qRT-PCR validation of the mRNA-SEQ analysis using  
818 Scr-KGN and KO-KGN cells (A) for 3up-regulatedp-regulated (SOD2, Pentraxin, IL6) and (B)  
819 3 down-regulated (COLL4a6, MMP11, CKB) differentially expressed genes.

820

821 **Supplemental data 5. Table. Comparison of DEGs with genes or proteins identifying as**  
822 **differentially expressed in PCOS women.**

823 Differentially regulated genes in KO-KGN cells were compared to genes or proteins present in  
824 a curated PCOS database ("PCOSbase" (<http://pcosbase.org>) (Afqah-Aleng *et al.*, 2017).  
825 Forty-two down-regulated and 14 up-regulated genes in KO-KGN cells were also found in the  
826 PCOSbase database providing support for considering these genes as dysregulated in PCOS



827 women. These 56 genes are involved in cytoskeleton reorganization, metabolism, lipid  
828 metabolism, and immunity.

829

830 **Supplemental data 6. BrDU assay performed on human primary granulosa-lutein cells**  
831 **exposed to AMPK inhibitors.** 20.000 cells/well were seeded and were exposed for 48h to  
832 AMPK inhibitor (Compound C and/or SBI-0206965). During the last 24h. cells were exposed  
833 to a pulse of BrDU (24h) (n=4). One-way ANOVA was performed with Dunnett post-hoc  
834 comparisons tests.

835

836 **Supplemental Figure 7. Gene expression performed on human primary granulosa-lutein**  
837 **cells exposed to AMPK inhibitor (Compound C) for 24hours (n=5).**

838

839 **Supplemental Figure 8. Expression of constitutively active AMPK rescues the KO-KGN**  
840 **deficient cells.** Gene expression performed on control KGN cells (KGN WT) . KO-KGN  
841 cells. and KO-KGN cells infected with an adenoviral construct encoding a constitutively  
842 active (CA-AMPK) (AdenoCA-AMPK-KO-KGN cells) for 48hours (n=3).

843

844 **Supplemental Figure 9. Representative picture of atretic and healthy follicle . \*: pyknotic**  
845 **cells in atretic follicle ; h: healthy follicle. Bar. 100 µm.**

846

847 **Table 1. Primers for quantitative Real-Time PCR**

Name.	Accession no	Forward primer	Reverse primer
$\alpha$ 1AMPK	NM_006251.6	TGCGTGTACGAAGGAAGAATCC	TGTGACTTCCAGGTCTTGGAGT
$\alpha$ 2AMPK	NM_006252.4	GGGTGAAGATCGGACACTACGT	TTGATGTTCAATCTTCACTTGT
interleukin 6 (IL6)	NM_001318095. 2	CCAGCTATGAACTCCTTCTC	GCTTGTTCCTCACATCTCTC
superoxide dismutase 2 (SOD2)	NM_001322819. 2	GACAAACCTCAGCCCTAACG	GAAACCAAGCCAACCCCAAC
Pentraxin 3	NM_002852.4	CGG TGC TAG AGG AGC TG	TAGCTGTTTCACAACCT
Collagen type IV alpha 6 chain (COLL4a6)	NM_001847	CACTATGCCAGGCGCAATG	CACACACAGAGCAGCGGCT
Matrix metallopeptidas e 11 (MMP11)	NM_005940.5	ATTTGGTTCTTCCAAGGTGCTCA GT	CCTCGGAAGAAGTAGATCTTGTT CT
Creatine kinase (CKB)	NM_001823	GCC TCA CTC AGA TCG AAA CTC	GGC ATG TGA GGA TGT AGC CC
Actin Beta (ACTB)	NM_001101.5	ACGGAACCACAGTTTATCATC	GTCCCAGTCTTCAACTATAACC
Eukaryotic Translation Elongation Factor 1 Alpha 1 (EEF1A1)	NM_001402.6	AGCAGACTTTGTGACCTTGCC	TCACATGAGACAGACGGTTGC

Glyceraldehyde -3-Phosphate Dehydrogenase (GAPDH)	NM_002046.7	ACGGATTTGGTCGTATTGGG	TGATTTTGGAGGATCTCGC
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850

851 **Table 2. Biological parameters of patients from control and PCOS groups.**852 Data are expressed as mean  $\pm$  S.E.M. (range)

853

	Control (n=8)	PCOS (n=9)	p
Age	30,63 $\pm$ 1,48 (26 - 37 )	29,56 $\pm$ 1,26 (22 - 34 )	NS
BMI	21,90 $\pm$ 0,83 (19 - 25 )	20,43 $\pm$ 0,70 (18 - 25 )	NS
Testosterone (mmol/L)	1,39 $\pm$ 0,57 (0 - 5 )	4,11 $\pm$ 1,48 (1 - 15 )	<0.05
AMH (ng/mL)	3.03 $\pm$ 0,74 (1 - 7 )	22,45 $\pm$ 6,02 (7 - 60 )	<0.0001
FSH (UI/L)	7,38 $\pm$ 1,33 (3 - 15 )	5,76 $\pm$ 0,35 (3 - 7 )	NS
LH (UI/L)	4.41 $\pm$ 0.32 (3 - 6 )	8.73 $\pm$ 1.44 (4 - 15 )	<0.05
E2 (ng/l)	40.74 $\pm$ 5.56 (25 - 73 )	46.01 $\pm$ 4.33 (32 - 72 )	N.S.
Cycle duration (days)	28.25 $\pm$ 0.87 (26 - 34 )	105.06 $\pm$ 23.86 (31 - 180 )	<0.001
number of total follicles (determined by ultrasound using Rotterdam criteria)	16.25 $\pm$ 1.94 (7 - 22 )	51.00 $\pm$ 5.86 (40 - 80 )	<0.001
number of mature oocytes retrieved	4.50 $\pm$ 0.71 (2 - 7 )	7.67 $\pm$ 1.71 (3 - 16 )	N.S.

854

855 **Supplemental data 5.**856 **Table with Genes down-regulated in AMPK KO granulosa cells and cited in PCOSbase**

857

protein name	gene_short_name	Log2 (FC)	p-adj	references
<b>Associated to cytoskeleton reorganization</b>				
collagen type IV alpha 6 chain	COL4A6	-2,811093816	6,41E-05	(Jansen <i>et al.</i> , 2004)
CXADR Ig-like cell adhesion molecule	CXADR	-2,22068001	0,003665591	(Wood <i>et al.</i> , 2005)
matrix metalloproteinase 11	MMP11	-2,191714855	0,002366685	(Oksjoki <i>et al.</i> , 2005)
collagen type IV alpha 3 chain	COL4A3	-1,812889506	0,01676443	(Wood <i>et al.</i> , 2003)
CD9 molecule	CD9	-0,938283962	0,015877884	(Oksjoki <i>et al.</i> , 2005)
lamin A/C	LMNA	-0,909314928	0,00524773	(Ma <i>et al.</i> , 2007)
formin 2	FMN2	-0,787480608	0,009844453	(Wood <i>et al.</i> , 2007)
spectrin repeat containing nuclear envelope	SYNE2	-0,783093104	0,006783443	(Diao <i>et al.</i> , 2004)
destrin, actin-depolymerizing factor	DSTN	-0,590541073	0,030849344	(Wood <i>et al.</i> , 2007)
S100 calcium-binding protein A4	S100A4	-1,895067365	1,57E-05	(Jansen <i>et al.</i> , 2004)
slit guidance ligand 2	SLIT2	-1,329733708	0,033698713	(Jansen <i>et al.</i> , 2004)
fascin actin-bundling protein 1	FSCN1	-1,238972477	0,02454077	(Jansen <i>et al.</i> , 2004)
secreted protein acidic and cysteine-rich	SPARC	-1,145819126	3,12E-09	(Diao <i>et al.</i> , 2004)
<b>Associated to metabolism</b>				
pyruvate dehydrogenase kinase 4	PDK4	-2,02806644	4,28E-07	(Diao <i>et al.</i> , 2004)
creatine kinase B	C.K.B.CKB	-1,821744257	0,000807503	(Jansen <i>et al.</i> , 2004)
ectonucleotide	ENPP1	-1,435662986	0,002736008	(Cortón <i>et al.</i> , 2007)
natriuretic peptide receptor 3	NPR3	-1,40238276	0,024227079	(Wood <i>et al.</i> , 2003)
ATPase Na <sup>+</sup> /K <sup>+</sup> transporting subunit beta 1	ATP1B1	-0,944986551	0,000111989	(Jansen <i>et al.</i> , 2004)
dimethylarginine dimethylaminohydrolase 1	DDAH1	-0,690831339	0,017201613	(Ma <i>et al.</i> , 2007)
transaldolase 1	TALDO1	-0,625534732	0,002736008	(Ma <i>et al.</i> , 2007)
malate dehydrogenase 2	MDH2	-0,528110753	0,036138334	(Ma <i>et al.</i> , 2007)
membrane bound O-acyltransferase domain	MBOAT2	-0,497114539	0,032578793	(Jansen <i>et al.</i> , 2004)
<b>Associated to lipid process</b>				
epoxide hydrolase 1	EPHX1	-1,100535918	5,50E-09	(Wood <i>et al.</i> , 2003)
carnitine palmitoyltransferase 1A	CPT1A	-1,075237703	7,29E-06	(Ma <i>et al.</i> , 2007)
peroxisomal proliferator-activated receptor	PPARG	-0,97258565	0,03948827	(Jansen <i>et al.</i> , 2004)
neurolysin	NLN	-0,80427469	0,036457112	(Wood <i>et al.</i> , 2007)
carnitine O-acetyltransferase	CRAT	-0,530129098	0,011647709	(Wood <i>et al.</i> , 2005)
enoyl-CoA delta isomerase 2	ECI2	-0,44686918	0,031734107	(Wood <i>et al.</i> , 2003)
<b>others process</b>				
major histocompatibility complex, class II,	HLA-DMB	-1,596002075	0,047066826	(Jansen <i>et al.</i> , 2004)
major histocompatibility complex, class II,	HLA-DRB1	-1,558923538	0,043282009	(Jansen <i>et al.</i> , 2004)
regulator of G protein signaling 4	RGS4	-2,097673072	1,77E-05	(Cortón <i>et al.</i> , 2007)
inhibitor of D.N.A.DNA binding 4,	ID4	-1,683876121	3,97E-09	(Wood <i>et al.</i> , 2005)
MIF4G domain containing	MIF4GD	-0,781064095	0,034780635	(Jansen <i>et al.</i> , 2004)
protein disulfide isomerase family A member	PDIA5	-0,719190616	0,000921574	(Skov <i>et al.</i> , 2007)
androgen receptor	AR	-0,692728486	0,033652953	(Panda <i>et al.</i> , 2016)
annexin A2	ANXA2	-0,677664138	0,00857582	(Ma <i>et al.</i> , 2007)
inositol-tetrakisphosphate 1-kinase	ITPK1	-0,672853363	0,011259896	(Jansen <i>et al.</i> , 2004)
adenosylhomocysteinase like 1	AHCYL1	-0,668608252	0,022208827	(Wood <i>et al.</i> , 2007)
down-regulator of transcription 1	DR1	-0,546407083	0,019436969	(Wood <i>et al.</i> , 2007)
transforming acidic coiled-coil containing	TACC1	-0,522493788	0,041172734	(Wood <i>et al.</i> , 2007)
N(alpha)-acetyltransferase 50, NatE catalytic	NAA50	-0,50865998	0,041238754	(Wood <i>et al.</i> , 2007)
chaperonin containing TCP1 subunit 3	CCT3	-0,49373307	0,023886345	(Ma <i>et al.</i> , 2007)

858

859

860 **Table with Genes up-regulated in AMPK KO granulosa cells and cited in PCOSbase**

protein name	gene_short_name	Log2 (FC) (in KO compared to control)	p-adj	references
<b><u>Associated to immunity process</u></b>				
caspase 4	CASP4	0,621825878	0,00940265	(Wood <i>et al.</i> , 2005)  (Zafari Zangeneh <i>et al.</i> , 2017)
interleukin 1 alpha	IL1A	1,769217139	0,01499045	
interleukin 6	IL6	2,144628118	8,78E-05	(Adams <i>et al.</i> , 2016)
C-X-C motif chemokine ligand 1	CXCL1	2,551937289	7,29E-06	(Adams <i>et al.</i> , 2016)
serpin family B member 2	SERPINB2	2,723673809	6,14E-06	(Adams <i>et al.</i> , 2016)
C-C motif chemokine ligand 20	CCL20	2,830678569	1,59E-10	(Adams <i>et al.</i> , 2016)
interleukin 1 beta	IL1B	3,43972107	2,69E-13	(Zafari Zangeneh <i>et al.</i> , 2017)
<b><u>expression of genes required for cumulus cell expansion</u></b>				
prostaglandin-endoperoxide synthase 2	PTGS2	1,698028668	0,00678344	(Schmidt <i>et al.</i> , 2014)
pentraxin 3	PTX3	1,921101334	0,00054765	(Jin <i>et al.</i> , 2020)
<b><u>others process</u></b>				
activin A receptor type 1B	ACVR1B	0,523757312	0,00738568	(Panda <i>et al.</i> , 2016)
follistatin	FST	0,865977418	0,01879147	(Norman <i>et al.</i> , 2001)  (Oksjoki <i>et al.</i> , 2005)
cytohesin 2	CYTH2	1,053382964	0,00664545	
superoxide dismutase 2	SOD2	1,164536069	0,02350078	(Borro <i>et al.</i> , 2007)
Uncoupling protein 3	UCP3	1,412930247	0,03166918	(Panda <i>et al.</i> , 2016)

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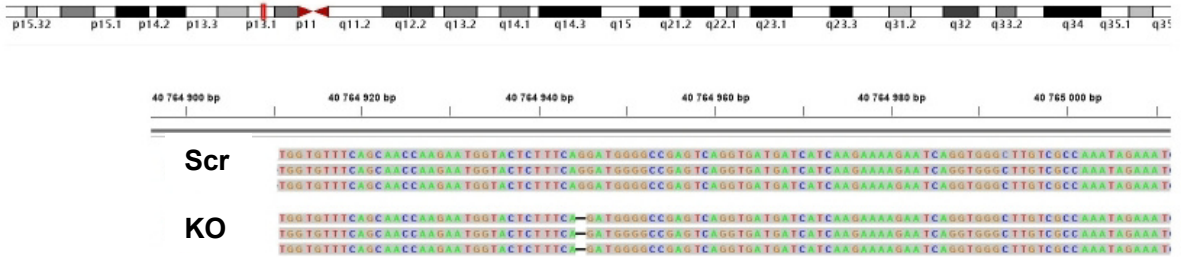
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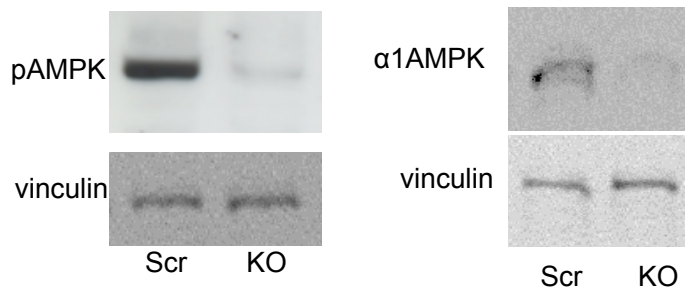
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# Figure 1

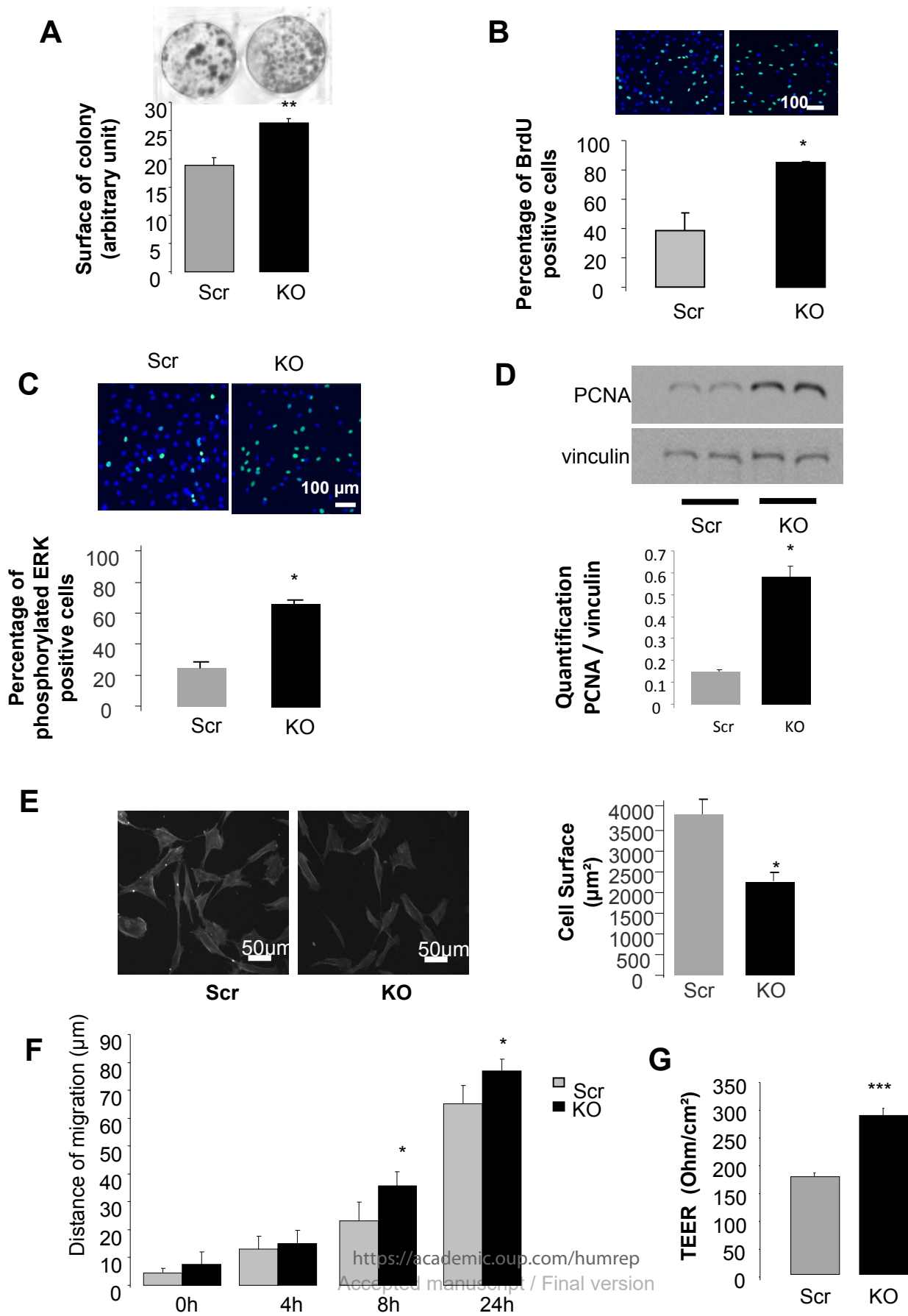
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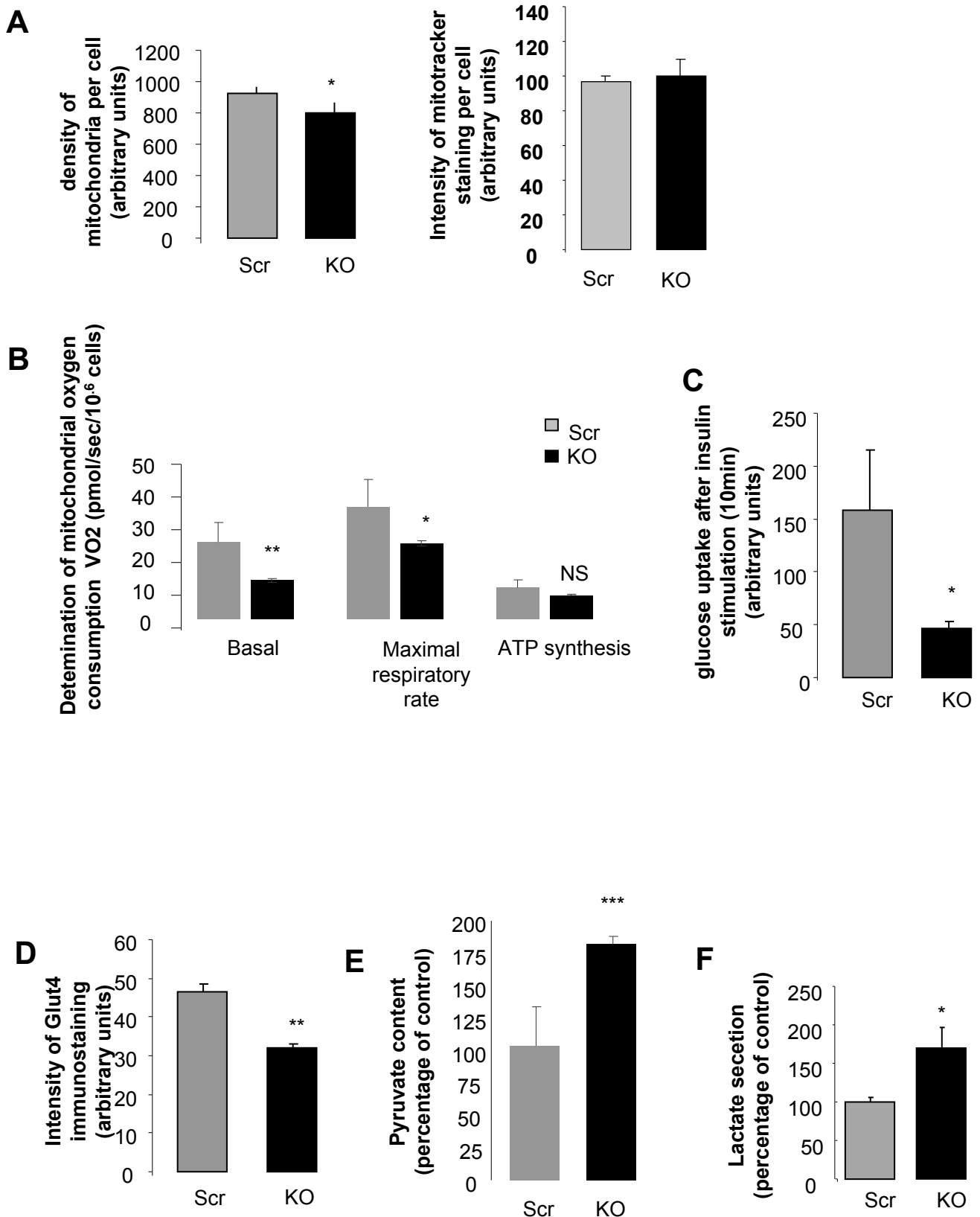


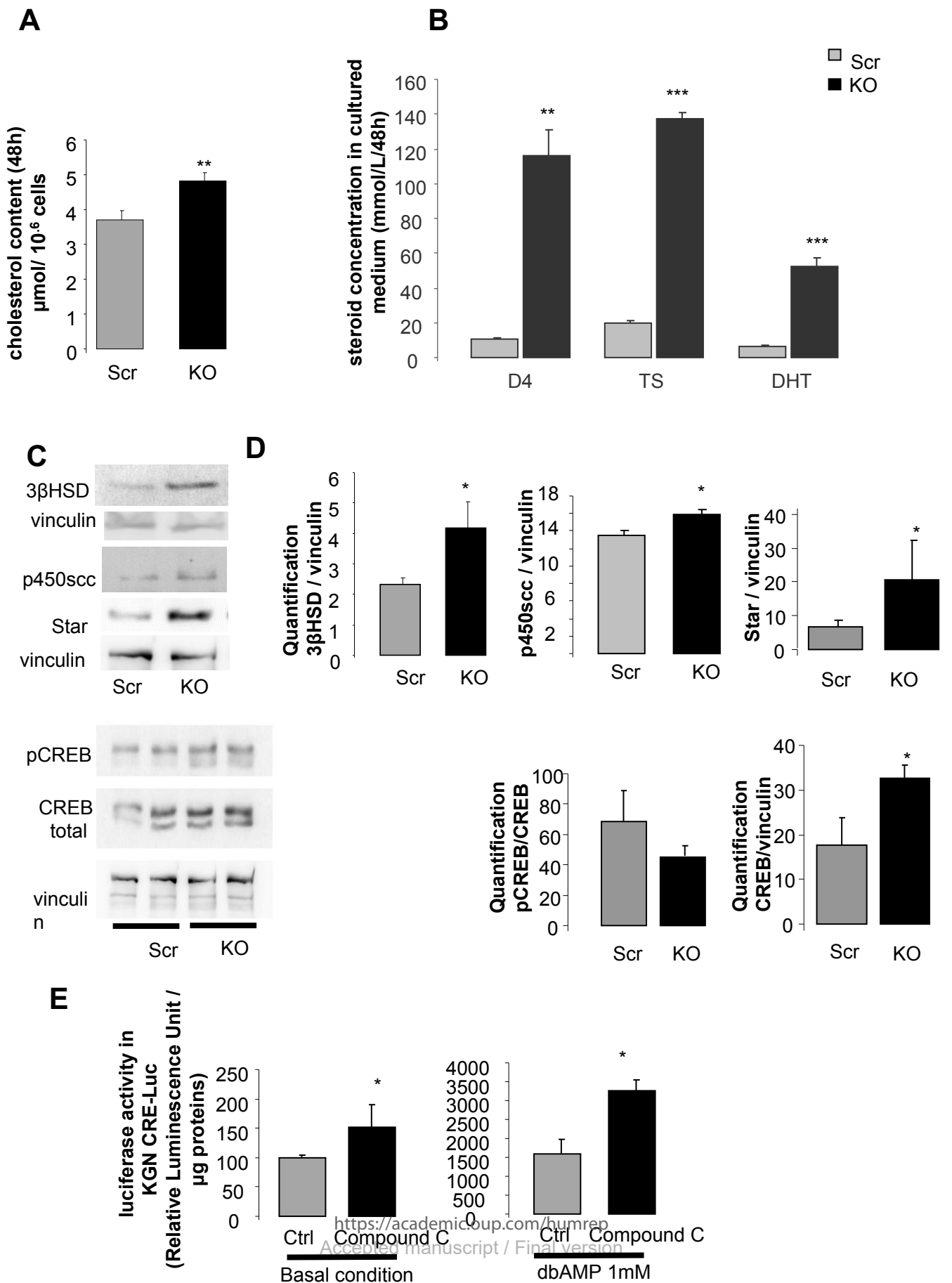
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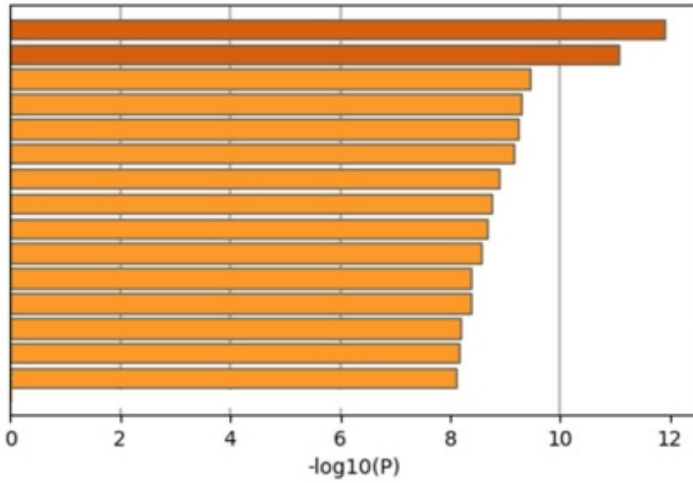
## Figure 2





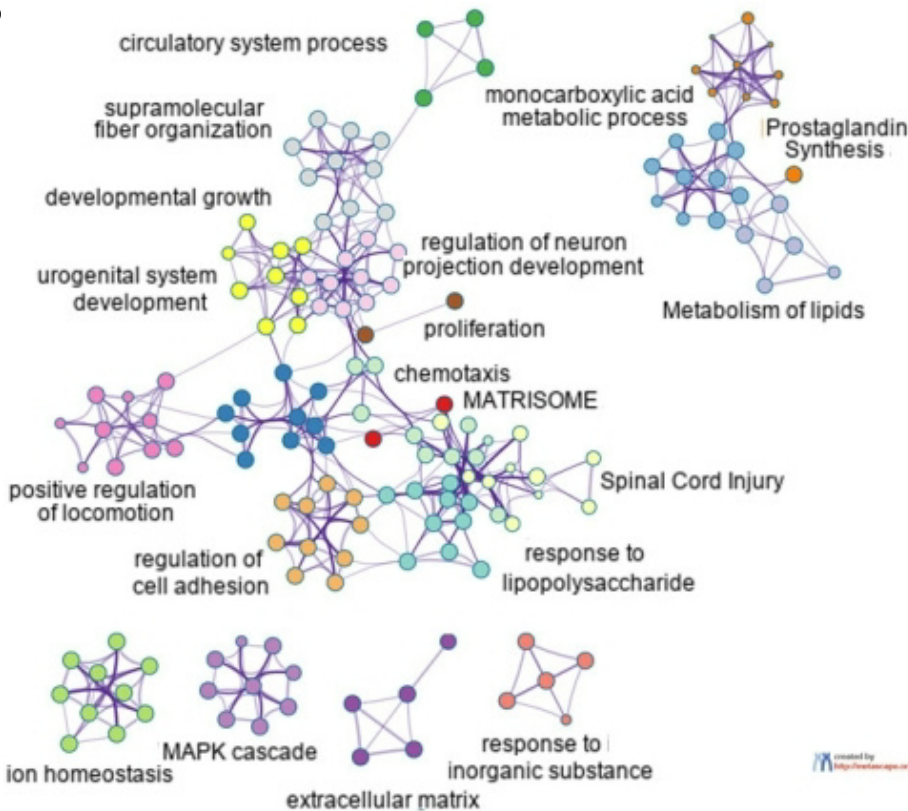


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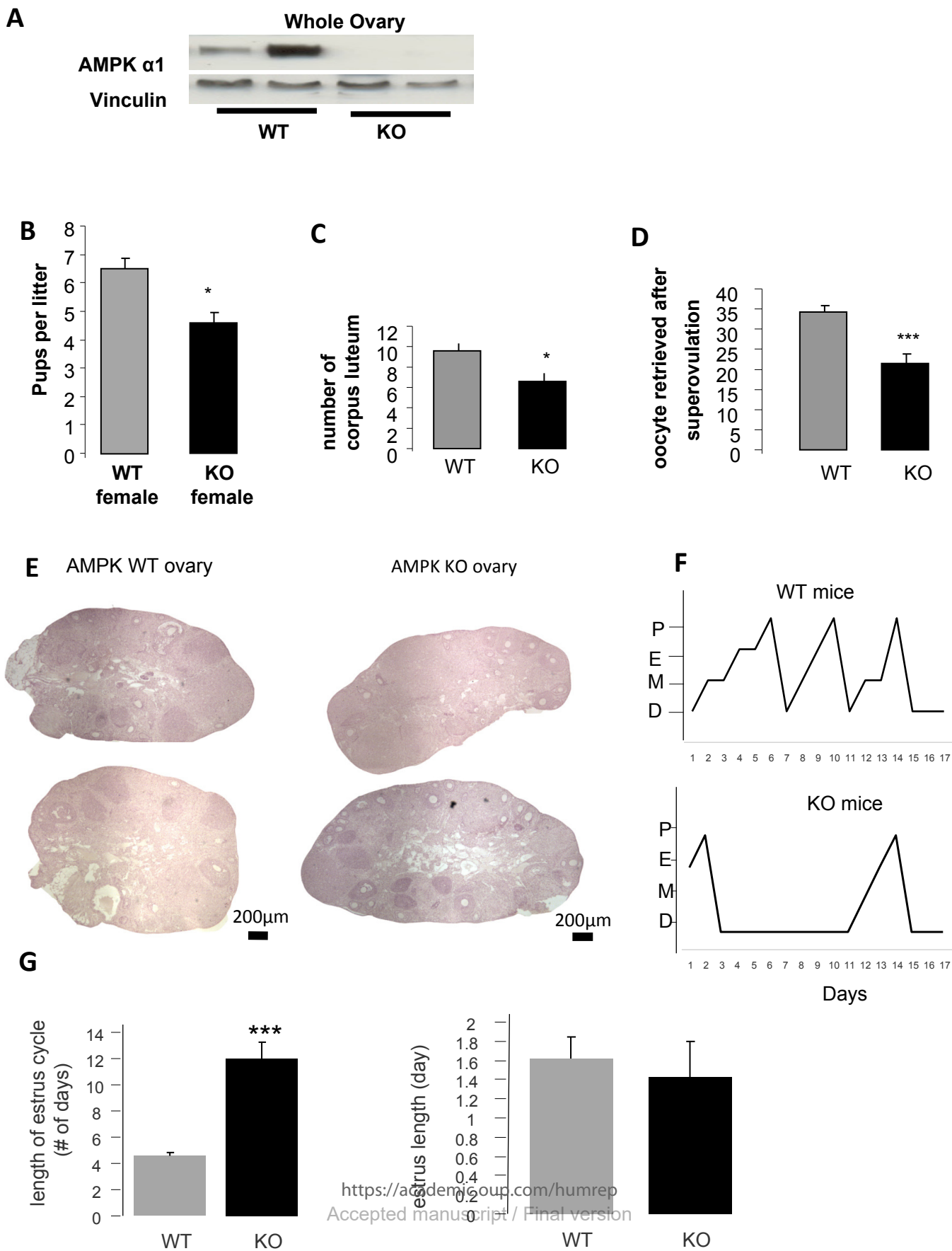


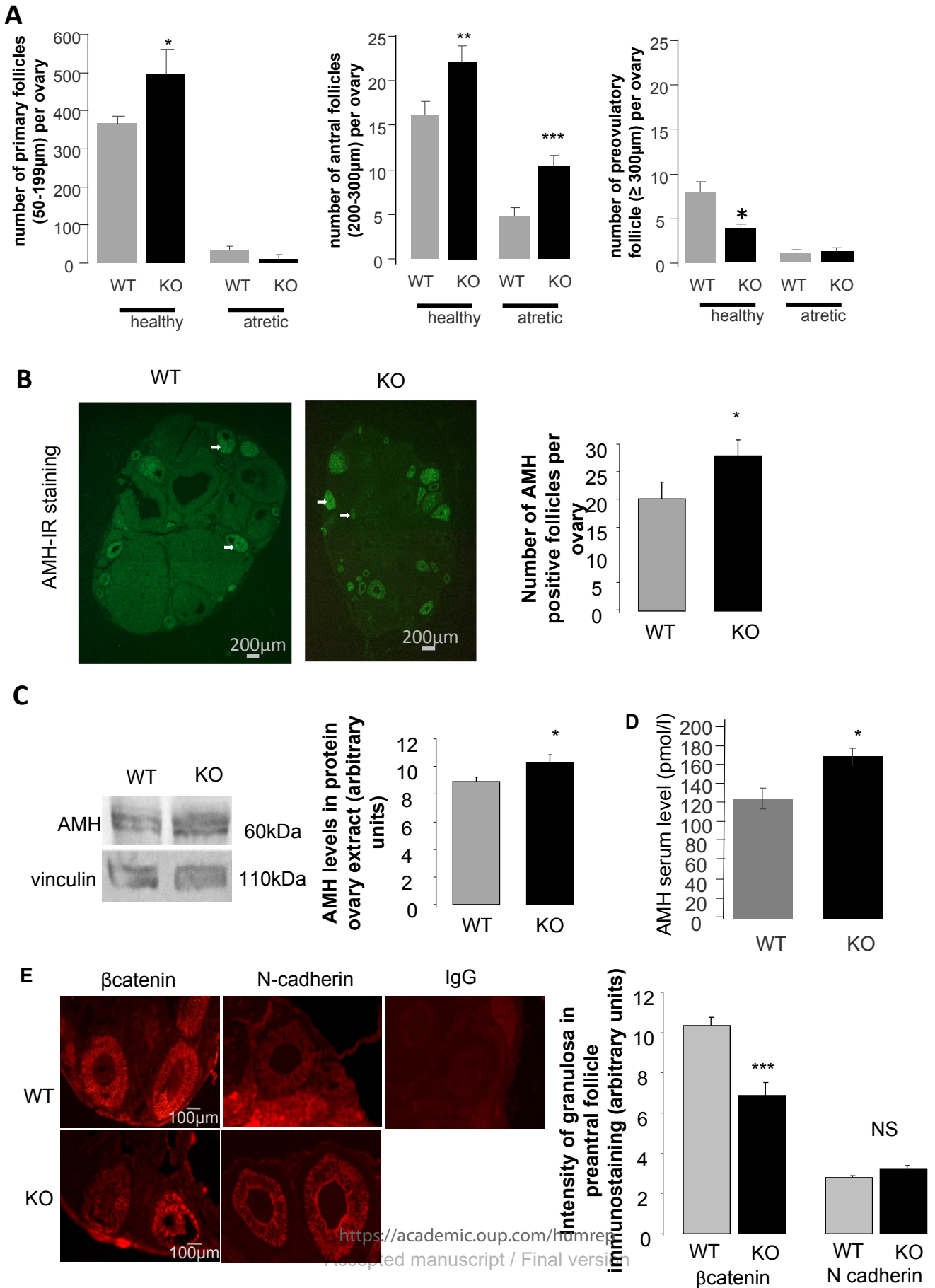
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 GO:0030198: extracellular matrix organization  
 WP98: Prostaglandin Synthesis and Regulation  
 GO:0048589: developmental growth  
 GO:0008285: negative regulation of cell population proliferation  
 GO:0001655: urogenital system development  
 GO:0051345: positive regulation of hydrolase activity  
 GO:0032496: response to lipopolysaccharide  
 WP2431: Spinal Cord Injury  
 R-HSA-556833: Metabolism of lipids  
 GO:0010035: response to inorganic substance  
 GO:0032787: monocarboxylic acid metabolic process  
 GO:0030155: regulation of cell adhesion

B

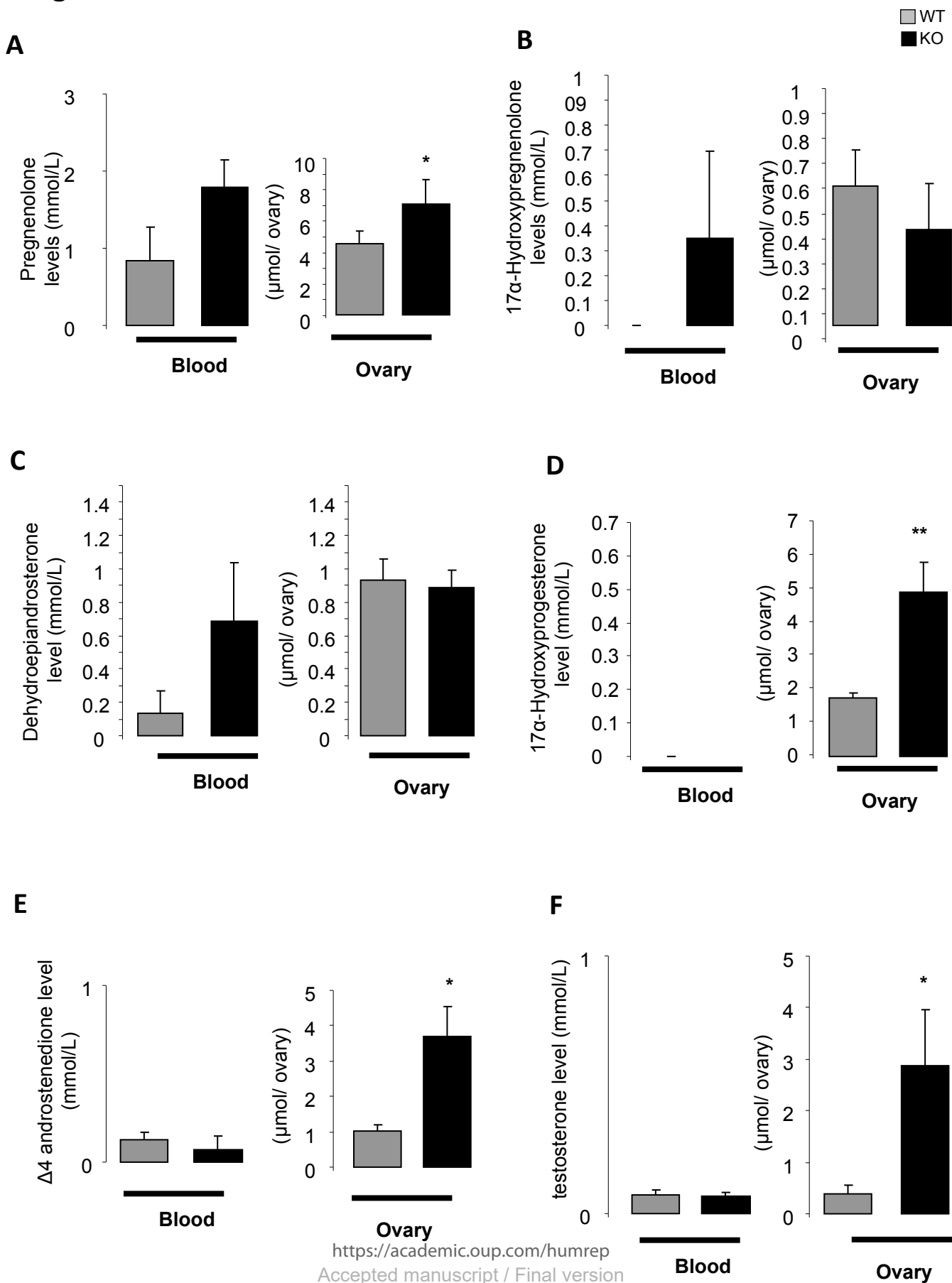


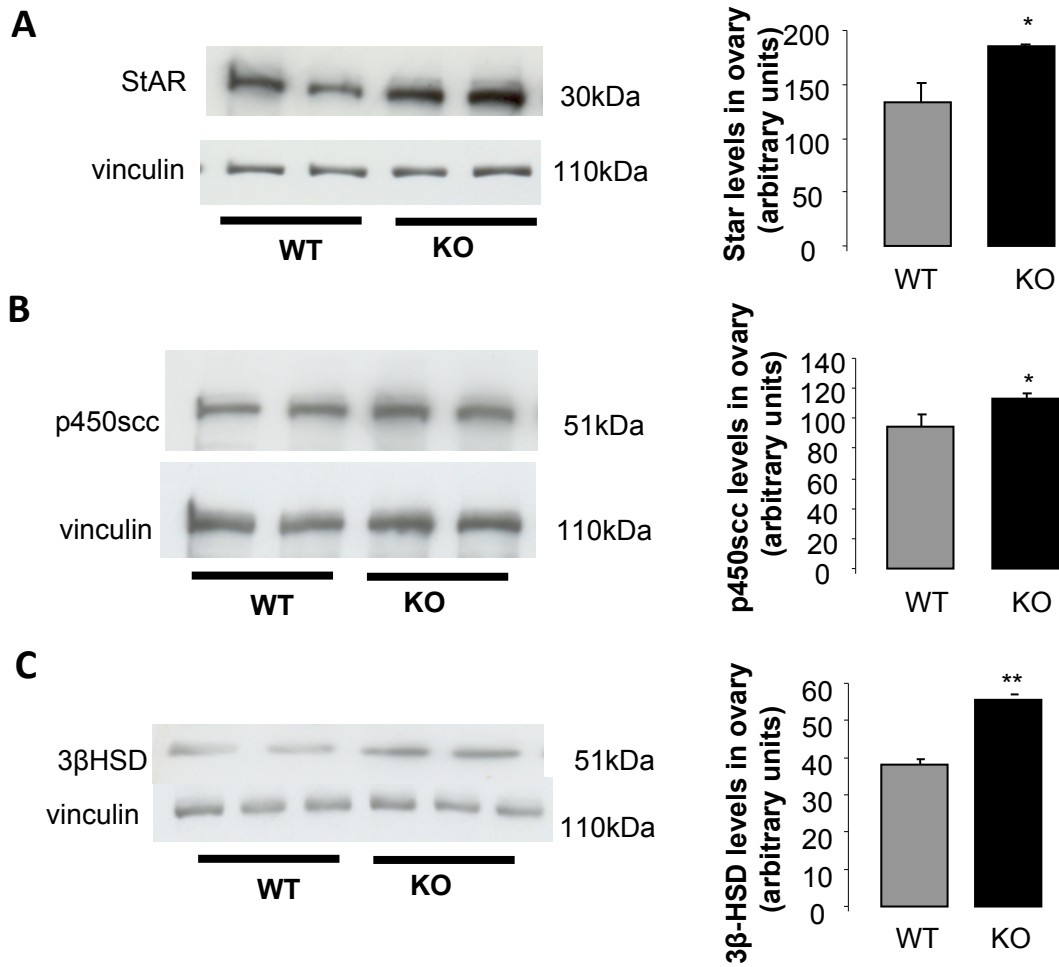
# Figure 6

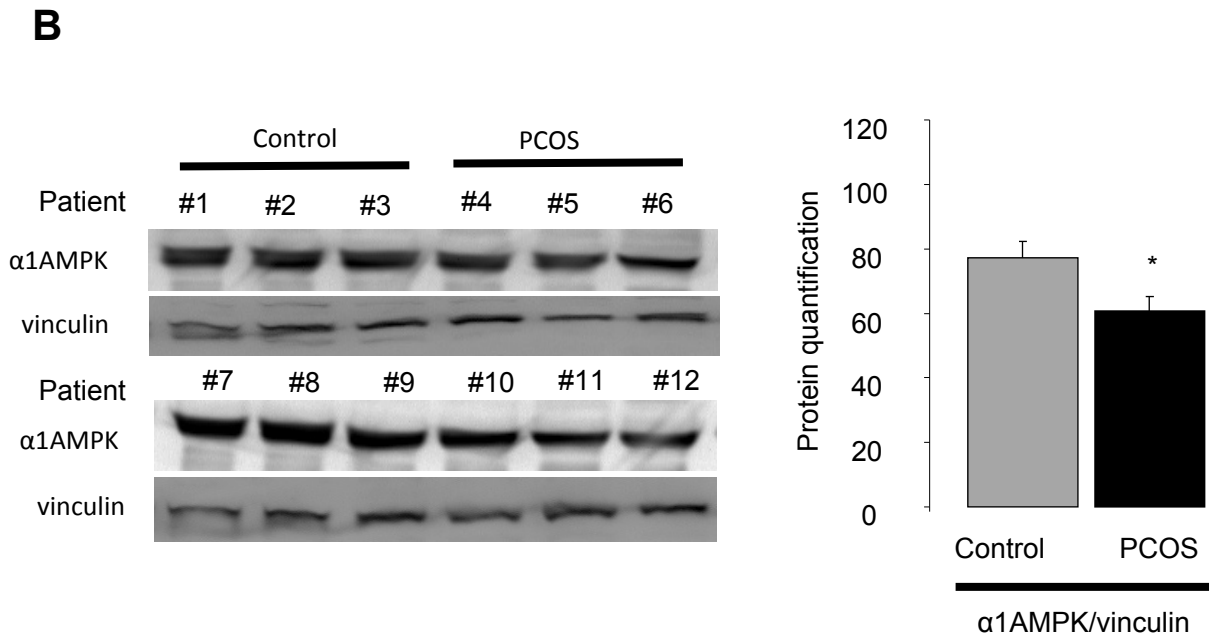
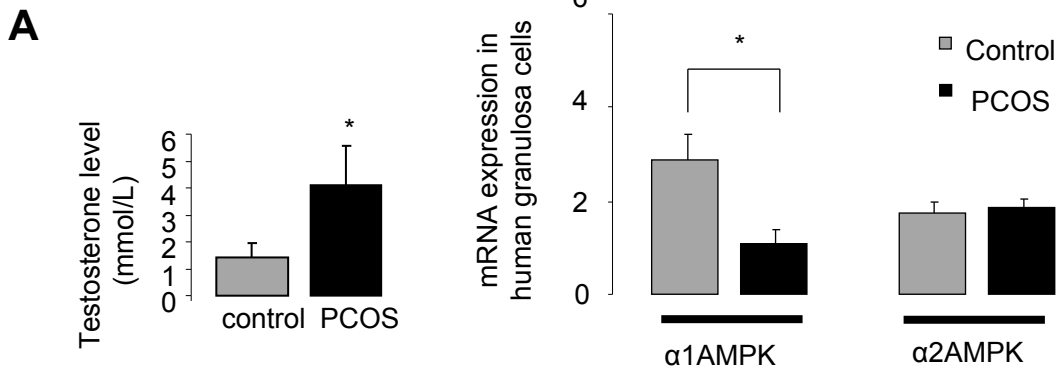


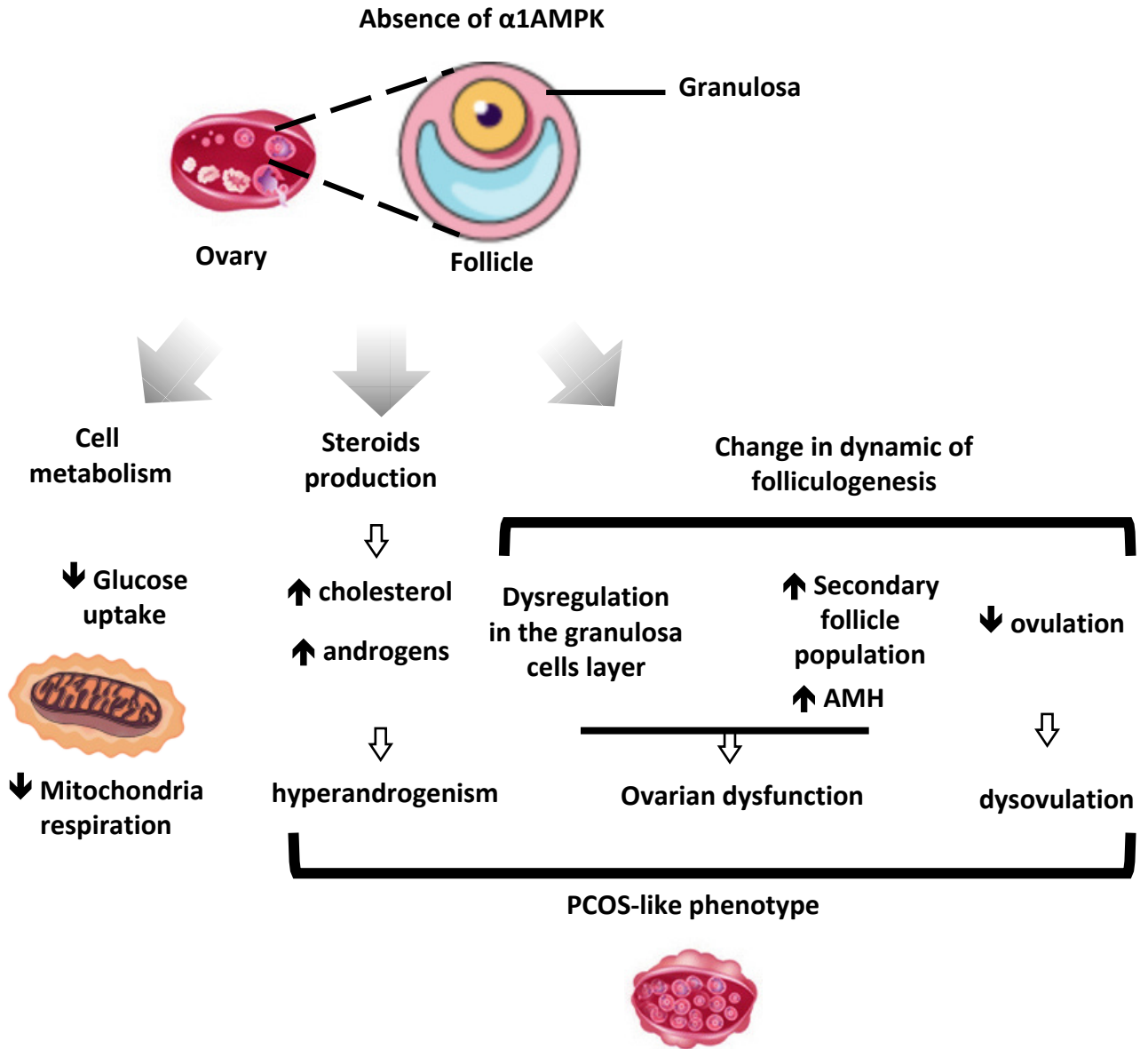




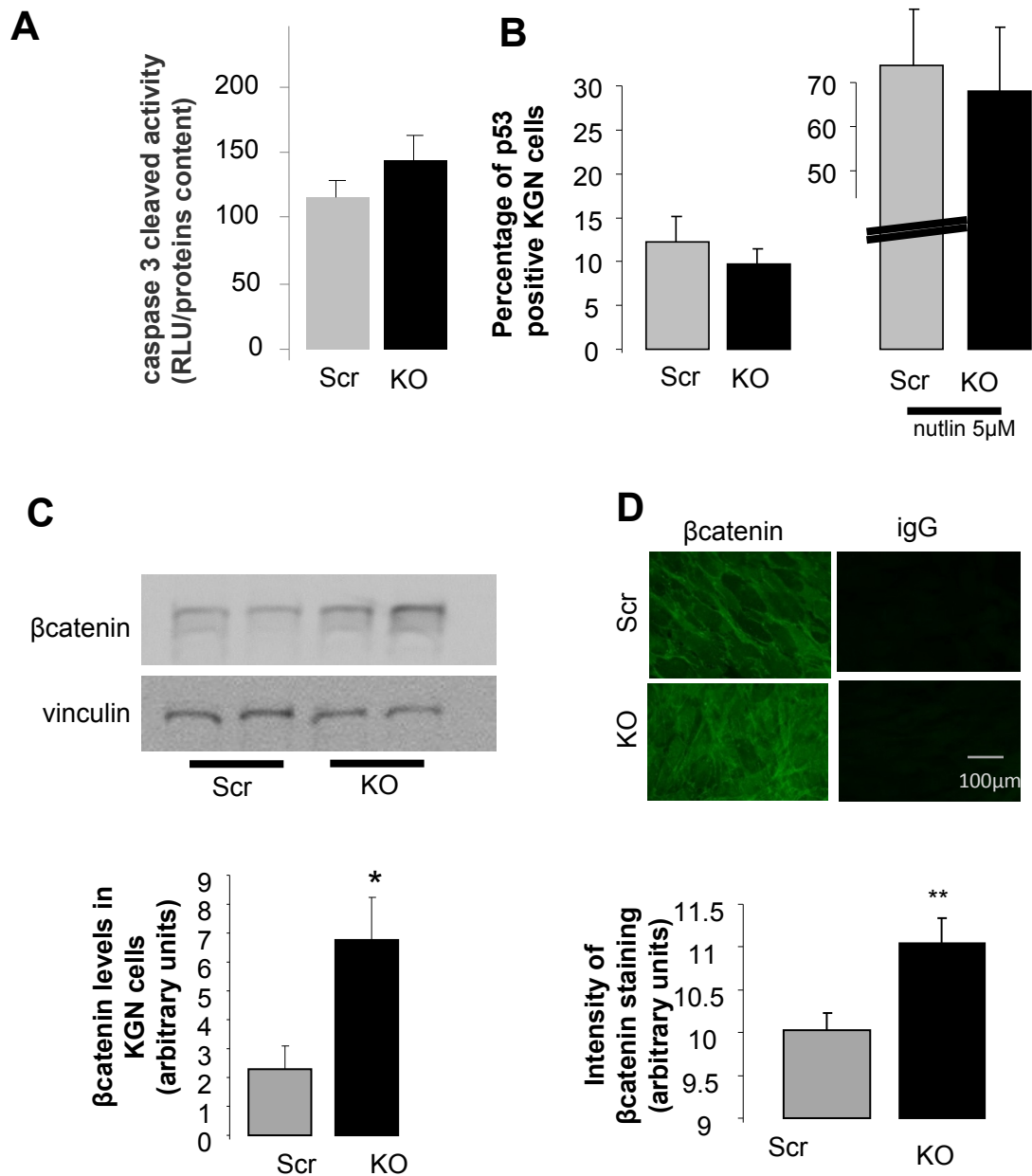


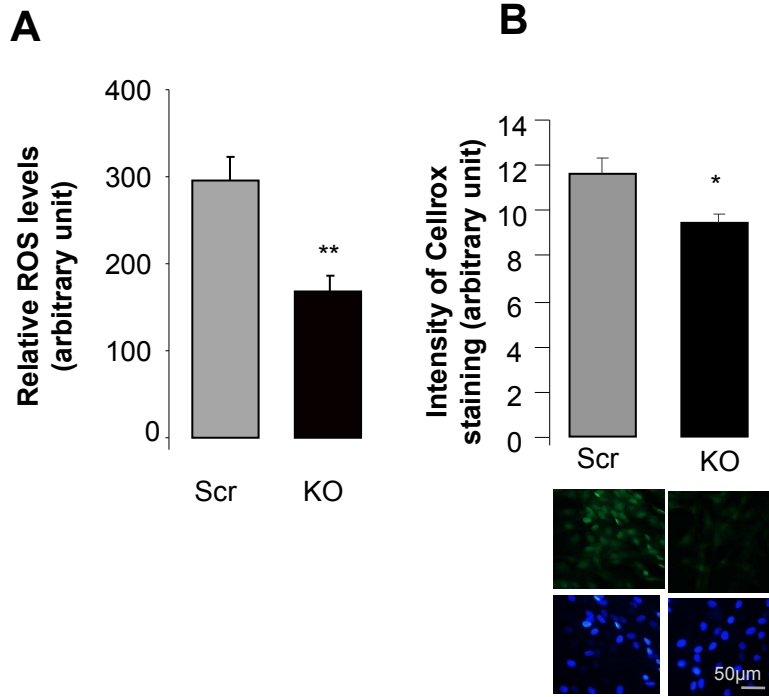




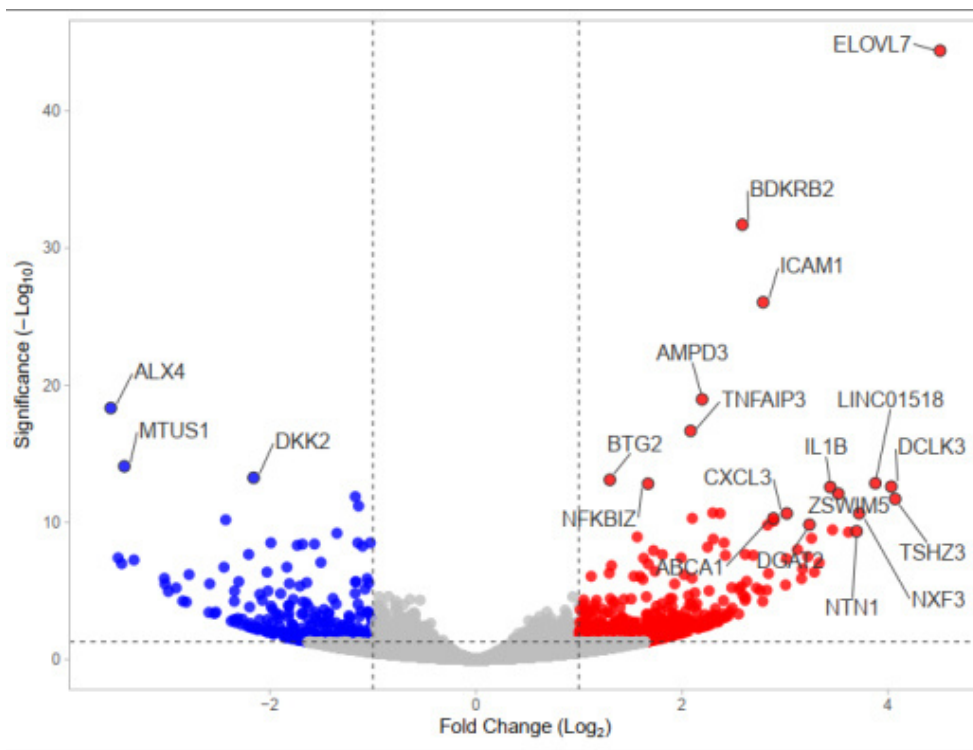


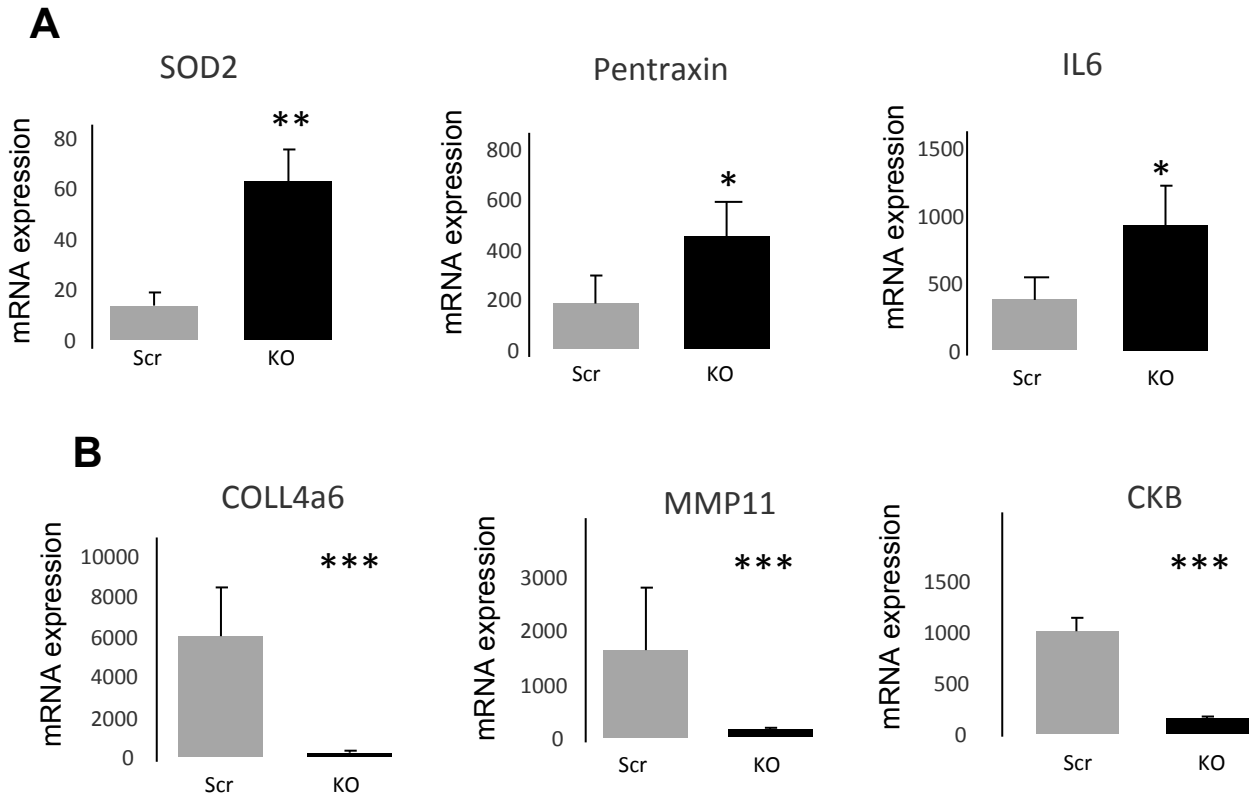
## Suppl Figure 1





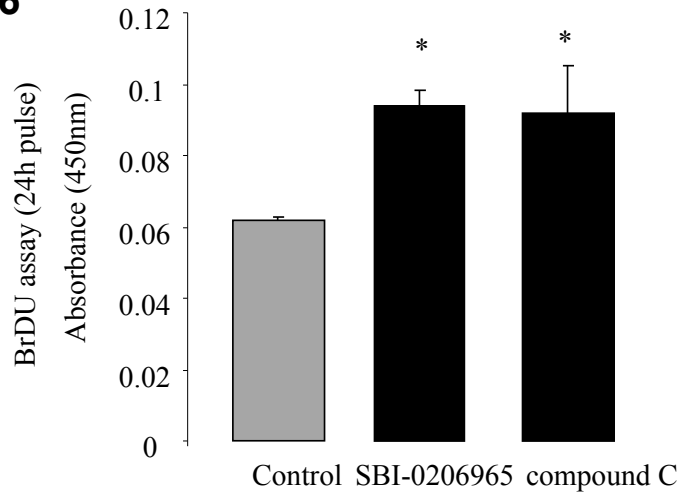
# Suppl Figure 3



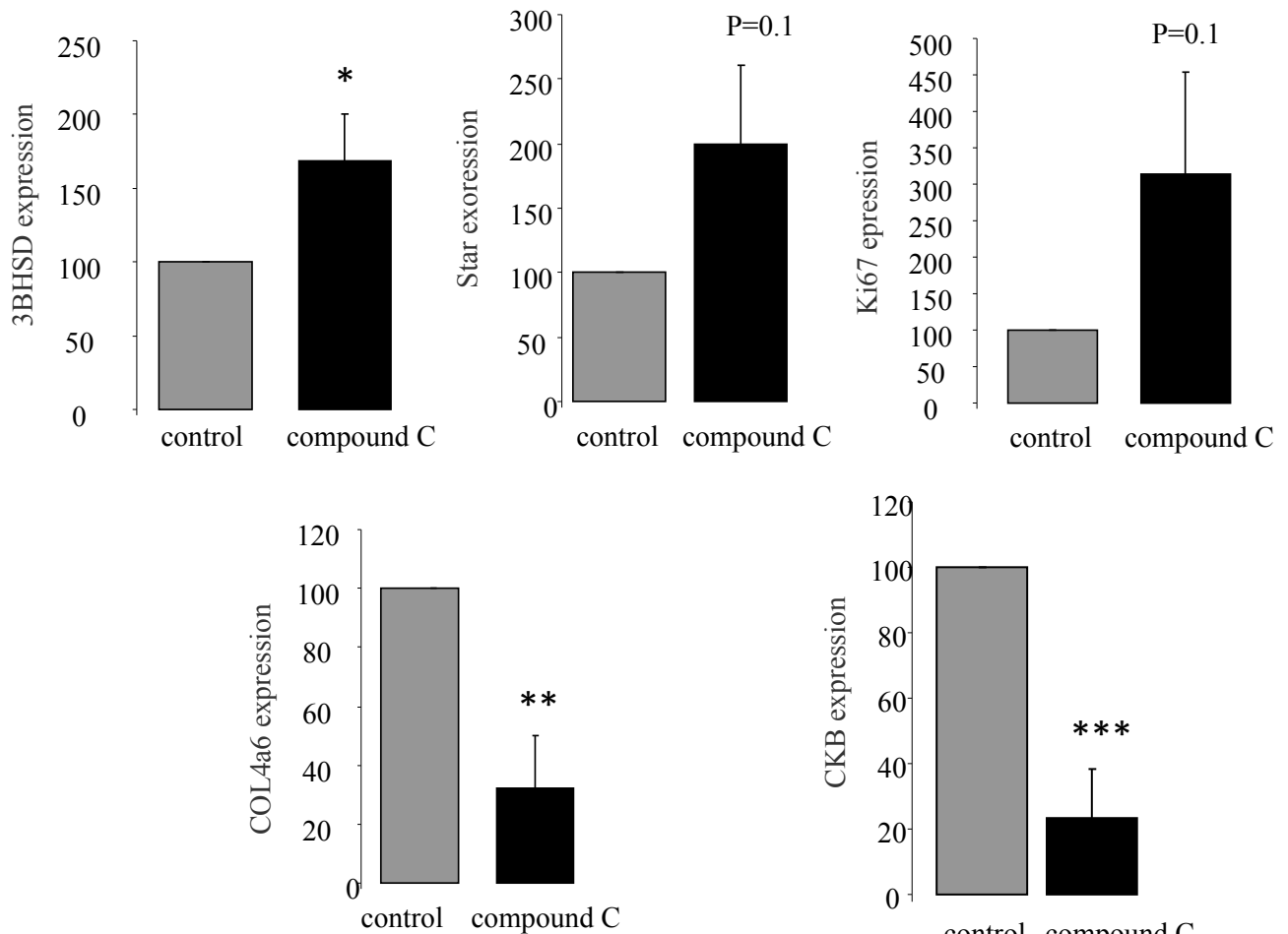


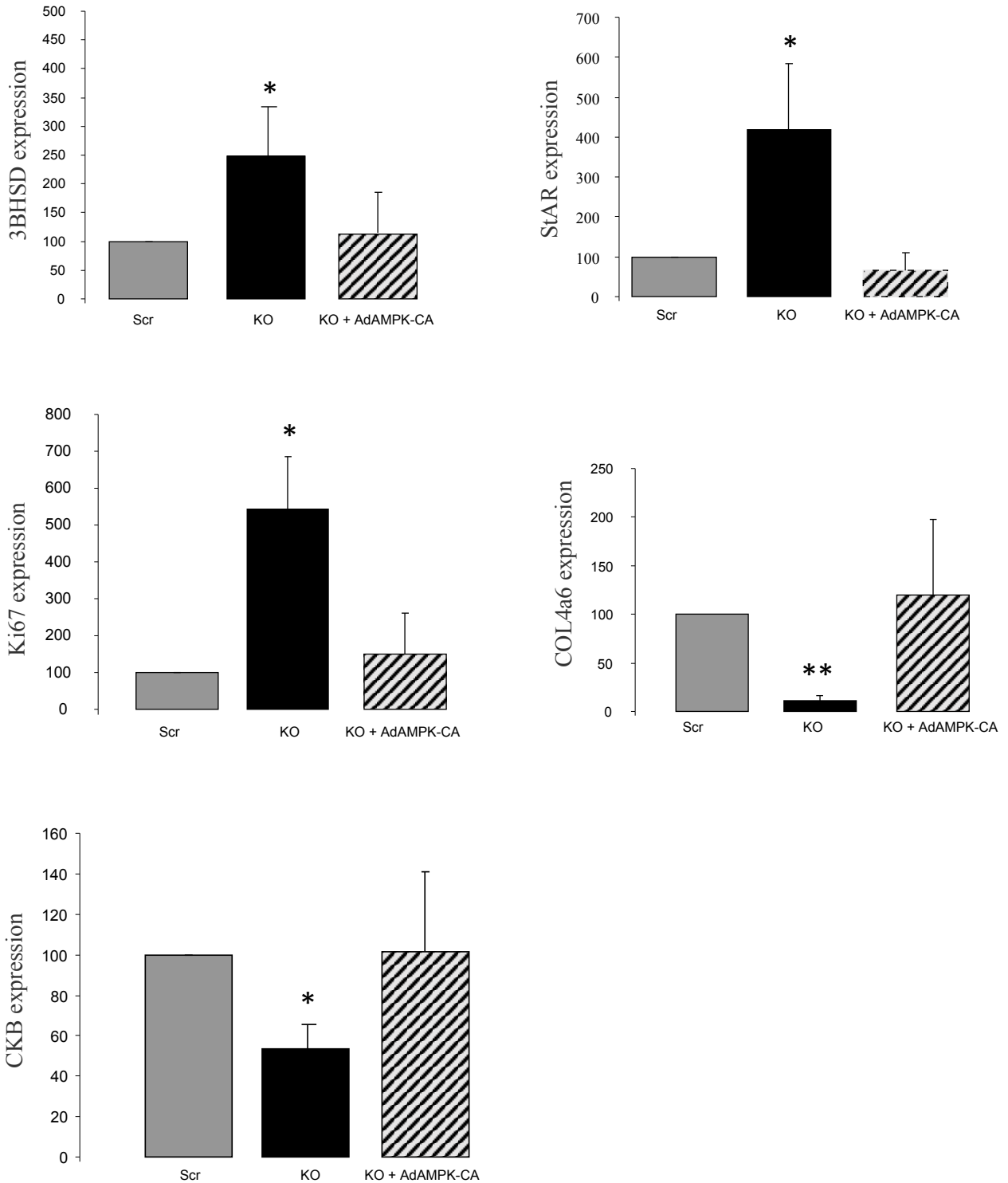


## Suppl Figure 6



## Suppl Figure 7



**Suppl Figure 8**

# Suppl Figure 9

