***ErbB4* regulates the oocyte microenvironment during folliculogenesis**

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27

28 **Abstract**

29

30 Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders leading
31 to infertility in women affecting reproductive, endocrine and metabolic systems. Recent genome-
32 wide association studies on PCOS cohorts revealed a single nucleotide polymorphism (SNP) in the
33 *ERBB4* receptor tyrosine kinase 4 gene, but its role in ovary development or during folliculogenesis
34 remains poorly understood. Since no genetic animal models mimicking all PCOS reproductive
35 features are available, we conditionally deleted *Erb4* in murine granulosa cells under the control of
36 *Amh* promoter. While we have demonstrated that *Erb4* deletion displayed aberrant ovarian function
37 by affecting the reproductive function (asynchronous oestrous cycle leading to few ovulations and
38 subfertility) and metabolic function (obesity), their ovaries also present severe structural and
39 functional abnormalities (impaired oocyte development). Hormone analysis revealed an upregulation
40 of serum luteinizing hormone, hyperandrogenism, increased production of ovarian and circulating
41 anti-Müllerian hormone. Our data implicate that *Erb4* deletion in granulosa cells leads to defective
42 intercellular junctions between the granulosa cells and oocytes, causing changes in the expression of
43 genes regulating the local microenvironment of the follicles. *In vitro* culture assays reducing the level
44 of *Erb4* via shRNAs confirm that *Erb4* is essential for regulating *Amh* level. In conclusion, our
45 results indicate a functional role for *Erb4* in the ovary, especially during folliculogenesis and its
46 reduced expression plays an important role in reproductive pathophysiology such as PCOS
47 development.

48

49 **Introduction**

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51 Human infertility is increasing worldwide. In Finland, for example, one woman out of six
52 cannot become pregnant naturally and will need fertility treatment (1). A better understanding of how
53 the signalling pathways affect and regulate fertility may provide clues for better treatment.

54 Dysfunction in the hypothalamo-pituitary-gonadal axis leads to reproductive disorders such
55 as hypogonadism, amenorrhea and polycystic ovary syndrome (PCOS). PCOS is a common
56 endocrinopathy affecting endocrine, reproductive, metabolic and psychological systems, and is the
57 most common case of anovulatory infertility (2). The disease is characterized by hyperandrogenism
58 associated with ovarian dysfunction or polycystic ovaries. Furthermore, PCOS patients show an
59 increased circulating luteinizing hormone (LH) concentration due to hyperandrogenism (3), and an
60 increased level of anti-Müllerian hormone (AMH) due to anovulation associated with a higher
61 number of growing follicles in the ovary (4,5). In rodent, AMH is needed to inhibit the initiation step
62 of the recruitment of primordial follicles as well as to affect FSH sensitivity of the non-selected
63 follicles at ovulation (6), whereas in human and nonhuman primates AMH does not have an action
64 during the primordial follicle development, but its role is to promote the growth and the survival of
65 the pre-antral follicle towards its maturation (7,8,9). Reproductive symptoms of PCOS are
66 accompanied with life-long metabolic syndromes such as insulin resistance, abdominal obesity, high
67 cholesterol and blood pressure, making PCOS a health threat beyond reproductive age as well (10).
68 According to one of the main hypotheses, the syndrome is the result of a prenatal hyperandrogenic
69 exposure that affects early embryonic development of the female offspring (11). This hypothesis has
70 been even furthermore highlighted by Tata *et al.* where excess prenatal AMH exposure alters GnRH
71 receptor signalling in the brain of the female embryos and also inhibits aromatase expression in the
72 placenta resulting in a reduced oestrogen level (12). However, the genetic and molecular mechanisms
73 behind the development of this disease have remained poorly understood mostly because of a lack of

74 animal models. To date, no animal models develop PCOS with all the defining characteristics of
75 human PCOS. Consequently, examining how the PCOS syndrome develops is particularly difficult.

76 Two recent genome wide association studies (GWAS) of Chinese and European cohorts
77 (13,14) have shown that a single nucleotide polymorphism (SNP) in epidermal growth factor receptor
78 4 (*ERBB4*) gene is associated with PCOS, indicating its role in regulating women fertility. *Erb4* is
79 a member of the epidermal growth factor receptor (Erb) family, which includes *Erb1* (also known
80 as *Egfr*), *Erb2*, *Erb3* and *Erb4*. *Erb4* differs from the other *Erb* genes since it can generate four
81 alternatively spliced isoforms (JMa-CYT1, JMa-CYT2, JMb-CYT1 and JMb-CYT2) with distinct
82 signalling properties (Fig. 1A). JM-a isoform contains a tumour necrosis factor-alpha converting
83 enzyme (TACE) cleavage site generating a an extracellular Erb4 ectodomain whereas JMB isoform
84 does not have it. Also, JM-a isoforms has a y secretase that releases the soluble intra-cellular domain
85 (ICD) which is translocated into the nucleus to regulate transcription (15). In mice, *Erb4* is necessary
86 for the development of the heart (16), kidneys (17), nervous system (18) and mammary glands (19).
87 It is further required for proper male fertility by affecting seminiferous tubule polarity. Improper
88 seminiferous tubule polarity may cause sperm motility defects (20) which was confirmed by a GWAS
89 study in Japanese men (21). Expression of *Erb4* can be observed in mouse granulosa cells (cumulus
90 and mural cells) during the ovulation process, and an induction of ovulation by human chorionic
91 gonadotropin (hCG), an analogue of LH, results in a decreased level of *Erb4* expression in the
92 ovaries of mice (22). In addition, no conditional knock-out mouse line has been generated before for
93 characterising Erb4 function in ovaries, we hypothesised that Erb4 plays a key role during selection
94 and maturation of the oocytes.

95 To define how the deletion of Erb4 in the ovary contributes to reproductive dysfunction, and
96 to identify the molecular mechanisms that are underlying the pathophysiology, we have performed
97 complementary *in vivo* and *in vitro* investigations, by generating a granulosa cells conditional *Erb4*
98 knock-out mice and knock-down granulosa cell line to probe the role of Erb4 in granulosa cells. The

99 results show that mis-regulated Erbb4 signalling affects ovarian function by regulating hormone and
100 Amh production, which may cause a PCOS-like phenotype.

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102

103

104 **Results**

105

106 **Cleavable Erbb4 JM-a Isoforms is expressed by the granulosa cells in the ovary**

107 *Erbb* (*Erbb*-1, -2, -3 and -4) receptor family and its ligands are expressed in the ovaries during
108 folliculogenesis (23), however little is known about *Erbb4* function during ovarian maturation and
109 which ligand binds to this receptor. Quantitative RT-PCR (qPCR) revealed that *Erbb4* mRNA was
110 expressed in the granulosa cells (GC) and oocytes of 1-month-old WT mice whereas *Erbb4*
111 expression is down-regulated in granulosa cells after ovulation (GC hCG) (Fig. 1B). The *Erbb4* gene
112 is expressed as four alternatively spliced isoforms with unique signalling characteristics (24). The
113 isoforms are composed of alternative extracellular juxtamembrane (JM) and intracellular cytoplasmic
114 (CYT) domains (Fig. 1A). The expression of all four *Erbb4* isoforms in the 1-month-old WT ovaries
115 was analysed by qPCR. *Erbb4* expression was detected and only isoforms *JM-a CYT-1* and *JM-a*
116 *CYT-2* were present, *JM-a CYT-2* was more abundant whereas *JM-b* was not observed (Fig. 1C). To
117 confirm the cell types that express *Erbb4*, *in situ* hybridization and immunohistochemistry methods
118 were carried out. In accordance with the real-time RT-PCR data (Fig. 1C), *Erbb4* expression was
119 detected in granulosa cells from primary to antral follicles (Fig. 1D, E). It is worth mentioning that
120 only the *ErbB4* JM-a isoforms can be proteolytically cleaved to produce a soluble intracellular
121 domain that can translocate into the nucleus and regulate transcription (25). Indeed, both nuclear and
122 cell membrane immunostaining for *ErbB4* was observed in the granulosa cells (Fig. 1F, insert higher
123 magnification of granulosa cells, black arrowheads depict the nuclear staining). Taken together, these
124 findings demonstrate that cleavable *Erbb4* isoforms are expressed during folliculogenesis. Several
125 ligands (Neuregulin, *Nrg1-4*; betacellulin, *Btc*; Heparin-binding EGF-like growth factor, *Hb-Egf*;
126 Epiregulin, *Ereg*) bind to *Erbb4* receptor to activate different pathways. We determined which
127 potential ligand binds to *Erbb4* in granulosa cells by quantifying their mRNA expression levels.
128 Granulosa cells expressed *Nrg1*, *Nrg4*, and *Hb-EGF*, whereas cumulus cells from super-ovulated

129 mice expressed *Ereg*, however, the expression of *Btc* was observed in both conditions (Fig. 1G).
130 Altogether, our data demonstrate that granulosa cells express *Erb4* specifically *JM-a* isoform and
131 *Erb4* interacts with selective receptors depending on the ovarian cycle suggesting an important
132 function in the ovaries.

133

134 ***Erb4* loss reduces cleavable *Erb4* JM-a CYT-2 Isoforms in the ovaries**

135 To dissect the *Erb4* function in granulosa cells of adult mice, we crossbred mice with floxed
136 *Erb4* gene (*Erb4^{Flox}*) (26) with mice expressing *Cre* recombinase under granulosa cell-specific
137 Müllerian inhibiting substance (*Amh*) gene (*AmhCre*) (27). We first confirmed the granulosa specific
138 expression by intercrossing *AmhCre* and *Rosa26LacZ* reporter mice (28). The results showed positive
139 LacZ staining only in the granulosa cells and not in the oocyte of the secondary follicle after birth
140 (Supplementary Fig. 1A). To verify whether *Erb4* expression has not been deleted in the other
141 tissues expressing *Amh* such as uterus and pituitary glands, qPCR was carried out. *Erb4* mRNA in
142 the uterus and pituitary glands were not affected by *Erb4* deletion whereas in the ovaries *Erb4*
143 mRNA was reduced by 3 folds (Fig. 2A). The weights of both organs did not change (Supplementary
144 Fig. 2A, B) and the histology of the uterus was similar in both genotypes (Supplementary Fig. 2C,
145 D).

146 Immunohistochemistry indicated that *Erb4* was indeed expressed in the granulosa cells of
147 the primary, secondary and pre-antral follicles of 17 days WT females (Fig. 2B-D). However, in the
148 granulosa cells of *Erb4^{Flox/Flox} ; AmhCre⁺* mice at the same age, *Erb4* expression was markedly
149 reduced in the cell membrane or in the nucleus (Fig. E-G) from the primary follicles stage onward
150 however its expression remained detected in the oocytes. The efficiency of the *AmhCre*-mediated
151 recombination varied, but on average the qPCR data showed an 81% reduction of *Erb4* expression
152 compared to the 2-month-old WT control females (data not shown). To further confirm the immuno-
153 histochemistry results, qPCR for *Erb4* isoforms was carried out. As expected, *JM-a* expression was

154 down-regulated by 5 folds. One fold change for *CYT-2* expression was observed in the *Erb4^{Flox/Flox}*
155 ; *AmhCre⁺* ovaries whereas *CYT-1* remained expressed (Fig. 2H, I). In *Erb4^{Flox/Flox}* ; *AmhCre⁺* mice,
156 Western blot analysis with ErbB4 antibody indicated a notable reduction in ErbB4 (180KD) and its
157 cleavable intracellular domain (ICD, 80KD) in the ovaries of the *Erb4^{Flox/Flox}* ; *AmhCre⁺* mice (Fig.
158 2J) in line with the qPCR and immunostaining data. Together, the results suggest that ErbB4, JM-a
159 CYT-1 isoform producing the cleavable ICD, is involved during ovarian development and
160 folliculogenesis.

161

162 **Reduced fertility and prolonged oestrous cycle in the *Erb4^{Flox/Flox}* ; *AmhCre⁺* females**

163 To analyse the fertility of the *Erb4^{Flox/Flox}* ; *AmhCre⁺* females, we repeatedly bred the females
164 with males of proven fertility. We found that *Erb4^{Flox/Flox}* ; *AmhCre⁺* females were sub-fertile, having
165 less progeny compared to the WT females, and giving birth to an average of six pups per litter
166 compared to eight pups for the WT mice followed for a period of 8 months (n = 10 for each genotypes,
167 $P < 0.001$, Fig. 3A, Supplementary Fig. 3A).

168 To explore if the regularity of the oestrous cycle is affected, we collected vaginal smears from
169 *Erb4^{Flox/Flox}* ; *AmhCre⁺* and WT females at the age of two, three and five months. The WT females
170 had a regular oestrous cycle of 3-5 days, whereas the *Erb4^{Flox/Flox}* ; *AmhCre⁺* females had an irregular
171 oestrous cycle ranging between 5 to 17 days with a significant decrease of time at metestrous (two to
172 three months-old) and an increase of time at diestrous (three to five months-old) ($P < 0.05$, n = 10 for
173 each genotype, Fig. 3B-D). To further investigate the changes in ovarian histology, sections of ovaries
174 from 5-week-old to 6 months--old *Erb4^{Flox/Flox}* ; *AmhCre⁺* females were evaluated. The *Erb4^{Flox/Flox}*
175 ; *AmhCre⁺* ovaries at P35 contained follicles with oocytes that were similar to the WT ovaries (Fig.
176 3E-F), but by the age of 3 months and 6 months significant differences were observed (Fig. 3G-J).
177 To further characterise the effect of the *Erb4* depletion in granulosa cells on the ovarian function,
178 follicle type counts were performed between *Erb4^{Flox/Flox}* ; *AmhCre⁺* and WT mice at the age of P35

179 and three months. Growing follicles are classified into four groups as primordial (oocyte surrounded
180 by squamous granulosa cell layer), primary (oocyte surrounded by a single layer of cuboidal
181 granulosa cells), secondary (oocyte surrounded by several layers of granulosa cells), and antral
182 follicles (follicle with a central located antrum) whereas the last class represents the soon ovulated
183 oocytes. Based on their morphology (29), the follicles were grouped into different categories:
184 primordial (dormant), primary, secondary, antral (growing) and atretic (dying). At P35, the
185 *Erb4^{Flox/Flox}; AmhCre⁺* ovaries displayed a higher number of secondary growing follicles compared
186 to the WT ovaries ($P < 0.05$, $n = 3$ *Erb4^{Flox/Flox}; AmhCre⁺*, $n = 3$ WT, Fig. 3K). The data gathered at
187 the age of three months, was in line with the P35 stage data ($P < 0.05$, $n = 7$ *Erb4^{Flox/Flox}; AmhCre⁺*,
188 $n = 3$ WT, Fig. 3L) showing that the oocytes were kept more in the stage of secondary follicles. The
189 number of atretic follicles did not changed between the genotypes. Furthermore, at the age of P35
190 and three-months, the *Erb4^{Flox/Flox}; AmhCre⁺* ovaries showed very few corpora lutea ($P < 0.001$, Fig.
191 3F-J, K, L) which is in line with the disrupted oestrous cycle and the lack of proper ovulation.
192 Collectively, our data demonstrate that the lack of Erb4 reduces fertility and induces changes during
193 the oestrous cycle, prolonging the late luteal phase.

194

195 **Reduction of *Erb4* delays ovulation by entrapping the oocytes in secondary follicle stage**

196 As we observed an arrest of some follicles at the secondary stage, we fully characterised the
197 follicle phenotype by histological examination at 25 days-old and 3 months. The nucleus of WT
198 follicles was condensed at both stages (Fig. 4A, D) whereas several *Erb4^{Flox/Flox}; AmhCre⁺* oocytes
199 showed decondensed chromatin or degenerating oocytes (Fig. 4B, C, E-I). Some of the oocytes that
200 failed to ovulate were entrapped in luteinised granulosa cells (Fig. 4J) suggesting that the follicle
201 maturation and oocyte activation were not synchronised properly. Further, we examined the number
202 of abnormal entrapped oocytes in cross-sections of ovaries from control and *Erb4^{Flox/Flox}; AmhCre⁺*
203 females at three months-old. The outcomes revealed the presence of 18.8 ± 7.8 abnormal oocytes per

204 cross-section in *ErbB4^{Flox/Flox}; AmhCre⁺* mice at 3 months-old compared to 8 ± 2.5 in WT females,
205 and normal oocytes were observed in both females ($P < 0.05$, $n = 7$ *ErbB4^{Flox/Flox}; AmhCre⁺*, $n = 3$
206 WT, Fig. 4K).

207 To further understand the impaired oocyte development, oocyte and granulosa cell markers
208 were quantified by qPCR. *Growth differentiation factor 9 (Gdf9)* was upregulated whereas the
209 expression of other oocyte markers *Homeobox protein NOBOX*, *Spermatogenesis- and oogenesis-*
210 *specific basic helix-loop-helix 1, proto-oncogene receptor tyrosine kinase (Nobox, Solh1, c-Kit)* was
211 reduced (Fig. 4L). Granulosa markers, *kit-ligand (Kit-L)* and *follistatin (Fst)*, were affected in
212 *ErbB4^{Flox/Flox}; AmhCre⁺* ovaries ($P < 0.05$, $n = 5$ *ErbB4^{Flox/Flox}; AmhCre⁺*, $n = 5$ WT, Fig. 4L). Our
213 results demonstrate that *ErbB4* and its signalling are involved in the ovulation via regulating the
214 oocyte activation.

215

216 ***ErbB4* reduction induces hyperandrogenism, LH hypersecretion and alters Cyp19a1 and** 217 **Hsd3b1 expression**

218 To further understand the underlying causes for the subfertility of the *ErbB4^{Flox/Flox}; AmhCre⁺*
219 females, we analysed the serum hormone concentrations of 3-month-old mice during different phases
220 of the oestrous cycle (proestrous, estrous, and metestrous) using gas chromatography-mass
221 spectrometry. The *ErbB4^{Flox/Flox}; AmhCre⁺* females displayed significantly irregular level of
222 hormones during the oestrous cycle compared to the WT females (30). Serum androstenedione level
223 is rising from oestrous to metestrus in WT ($P < 0.05$, $n = 3$ WT, Two way Anova followed by Tukey's
224 multiple comparisons test to compare the phases of the estrous cycle within the group) whereas in the
225 *ErbB4^{Flox/Flox}; AmhCre⁺* females the level was not increasing (Fig. 5A). Serum progesterone level
226 increased steadily from proestrus to metestrus in WT females ($P < 0.05$, $n = 3$ WT, Two way Anova
227 followed by Tukey's multiple comparisons test to compare the phases of the estrous cycle within the
228 group) whereas in the *ErbB4^{Flox/Flox}; AmhCre⁺* females the level reduced suddenly from estrous to

metestrus (Fig. 5B). The oestradiol levels in both genotypes have the same pattern (Fig. 5C). Testosterone level increased from proestrus and peaked in oestrous-metestrus in *ErbB4^{Flox/Flox};AmhCre⁺* mice ($P < 0.05$, $n = 3$ *ErbB4^{Flox/Flox};AmhCre⁺*, Two way Anova followed by Tukey's multiple comparisons test to compare the phases of the estrous cycle within the group) whereas the opposite was observed in WT mice (Fig. 5D). The results were in line with the observed abnormal folliculogenesis and the lack of proper ovulation. Analysis of gonadotropin concentrations revealed that LH was increased in 2-, 3- and 6-month-old mice ($P < 0.05$, Fig. 5E) and FSH was not affected compared to the age-matched WT littermates (Fig. 5F).

We also analysed the *LH receptor (Lhcgr)* mRNA localisation in diestrous cycle phase ovaries. In the WT ovaries, *Lhcgr* was localised in the theca cells of the antral follicles and corpus luteum (Fig. 5G), whereas in the *ErbB4^{Flox/Flox};AmhCre⁺* ovaries abundant expression was found in the mural granulosa cells of the antral follicles, in the theca cells, in the corpus luteum and in the interstitium (Fig. 5H).

To further analyse the biosynthesis of steroidogenic hormones in the ovary, we performed *in situ* hybridisation experiments on steroidogenic enzyme genes *3 beta-hydroxysteroid dehydrogenase (Hsd3b1)*, *hydroxysteroid 17-beta dehydrogenase 1 (Hsd17b1)* and *aromatase (Cyp19a1)*. LH is a potent inducer of *Hsd3b1* mRNA in granulosa cells during the ovulatory period (31). In the WT ovaries, *Hsd3b1* was expressed in the theca cells (arrows) and granulosa cells (yellow arrowheads) of growing follicles (pre-antral (PA) and secondary (SF) follicles) and distributed in the functional luteal cells, corpus luteum (CL) (Fig. 5I, K). We found a reduced expression of the *Hsd3b1* gene in the *ErbB4^{Flox/Flox};AmhCre⁺* granulosa cells (Fig. 5J, L). *Hsd17b1* is an essential enzyme for the synthesis of testosterone from androstenedione and for converting oestrone (E1) into oestradiol (E2). *Cyp19a1* is the enzyme converting testosterone into E2. In the WT ovaries *Hsd17b1* and *Cyp19a1* are expressed in the granulosa cells of growing follicles, are down-regulated in luteinizing granulosa cells, and not present in the corpora lutea (32). As expected, the *ErbB4^{Flox/Flox};AmhCre⁺* females also had abundant *Hsd17b1* and *Cyp19a1* transcripts in the granulosa cells (Fig. 5M, N). Our data

254 demonstrate that the reduction of *Erb4* induces hormone level imbalance in *Erb4^{Flox/Flox}; AmhCre⁺*
255 females mice.

256

257 ***Erb4* reduction promotes structural changes of theca cells and disturbs cell adhesion in**
258 **granulosa cells**

259 To understand more deeply why the follicle growth was not developing properly, we looked
260 at the ultrastructure of antral follicles by electron microscopy. In the ovaries of *Erb4^{Flox/Flox};*
261 *AmhCre⁺* females, the granulosa/ cumulus cells were less connected to each other than in the ovaries
262 of the WT mice. Increased extra-cellular distance was observed in the *Erb4^{Flox/Flox}; AmhCre⁺* ovaries
263 compared to the WT ones and the quantification was significant ($P < 0.05$, $n = 3$ *Erb4^{Flox/Flox}*
264 *;AmhCre⁺*, $n = 3$ WT, Fig. 6A-C, E,G). The theca cell layer in the *Erb4^{Flox/Flox}; AmhCre⁺* females
265 was thicker and the theca cells contained more intra-cytoplasmic vesicles than in the WT ovaries ($P <$
266 0.05 , $n = 3$ *Erb4^{Flox/Flox};AmhCre⁺*, $n = 3$ WT, Fig. 6D-F, H). Finally, a closer look was taken to
267 check the oocytes at the transzonal projections (TZPs) and microvilli. TZPs consist of granulosa cell
268 extensions to reach another granulosa cells, and microvilli are extension from the zona pellucida of
269 the oocyte terminating on the opposite cumulus cells. TZP enable the formation of the intercellular
270 gap junctions between the granulosa cells required for follicular development (33). Both were reduced
271 in *Erb4^{Flox/Flox}; AmhCre⁺* females compared to the WT females (Fig. 7I, J; red arrowheads for TZP
272 and black arrows for microvilli).

273 We further examined the cell adhesion markers for the granulosa cells in the *Erb4^{Flox/Flox};*
274 *AmhCre⁺* ovaries by analysing the expression of collagen IV and Connexin 43 (Gja1 or Cnx43) with
275 immunohistochemistry. Type IV collagen (Col4A1) was localised in the theca cells, around the
276 mature follicles in the WTs (Supplementary Fig.4A) as previously published (34), but in the
277 *Erb4^{Flox/Flox}; AmhCre⁺* oocytes Col4A1 expression was reduced (Supplementary Fig.4B). Gja1, a
278 marker for gap junctions between granulosa cells, is crucial for proper folliculogenesis, and absence

279 or decreased of *Gja1* expression in the follicle leads to impaired fertility by arresting the follicle into
280 the early preantral stage (35). In addition, gap junction marker *Gja1* expression was reduced in the
281 *Erb4^{Flox/Flox}; AmhCre⁺* follicles (Supplementary Fig.4D compared to the WT follicles,
282 Supplementary Fig.4C). β -catenin (*Ctnnb1*) is involved in mediating intercellular junctions and is
283 predominantly expressed in the plasma membrane of granulosa cells and zona pellucida (36). While
284 β -catenin was mainly expressed in the granulosa cells and zona pellucida of the WT mice, the
285 expression pattern was expanded to cover most of the granulosa cells and the surfaces of their plasma
286 membrane in the *Erb4^{Flox/Flox}; AmhCre⁺* follicles (Supplementary Fig.4E,F). These results show that
287 the reduction of *Erb4* alters the theca cell ultra-structure and affects the adhesion between the oocyte
288 and granulosa cells.

289

290 ***Erb4* reduction promotes obesity and adipocyte hypertrophy**

291 A body weight comparison showed that the *Erb4^{Flox/Flox}; AmhCre⁺* females increased than
292 the WT control females starting from 6 weeks until the age of 6 months ($P < 0.05$; 6 weeks $n = 11$
293 *Erb4^{Flox/Flox}; AmhCre⁺*, $n = 10$ WT; 3-months $n = 10$ *Erb4^{Flox/Flox}; AmhCre⁺*, $n = 10$ WT; 6-months
294 $n = 36$ *Erb4^{Flox/Flox}; AmhCre⁺*, $n = 31$ WT, Fig. 7A). In contrast, the *Erb4^{Flox/Flox}; AmhCre⁺* females
295 had an increased ovarian weight compared to the age-matched WT females at the age of 6 weeks and
296 six months (6 weeks and 6 months $P < 0.05$; 6 weeks $n = 9$ *Erb4^{Flox/Flox}; AmhCre⁺*, $n = 10$ WT; and
297 6 months $n = 36$ *Erb4^{Flox/Flox}; AmhCre⁺*, $n = 31$ WT, Fig. 7B). The ratio of ovarian weight to body
298 weight of *Erb4^{Flox/Flox}; AmhCre⁺* confirmed that the ovarian weight was heavier than in WT females
299 ($P < 0.001$ at 6 weeks $n = 9$ *Erb4^{Flox/Flox}; AmhCre⁺*, $n = 10$ WT; and $P < 0.05$ at 6 months $n = 36$
300 *Erb4^{Flox/Flox}; AmhCre⁺*, $n = 31$ WT, Fig. 7C).

301 As the *Erb4^{Flox/Flox}; AmhCre⁺* female mice increased their weight, we investigated the effect
302 of *Erb4* knockout on serum insulin levels and adipose tissue. We analysed the basal non-fasting
303 insulin serum levels at the age of 3 months in the *Erb4^{Flox/Flox}; AmhCre⁺* and WT females. The

304 *Erb4^{Flox/Flox}; AmhCre⁺* mice presented a significantly higher basal non-fasting insulin concentration
305 than the WT females ($P < 0.05$, Fig. 7D). As *Erb4^{Flox/Flox}; AmhCre⁺* mice presented a slightly higher
306 body mass (Fig. 7B), gonadal adipose tissues from *Erb4^{Flox/Flox}; AmhCre⁺* and WT females were
307 stained with haematoxylin and eosin, and the adipocyte diameter was measured. A slight increase in
308 the adipocyte diameter was observed but the leptin level remained the same (Fig. 7E-G, and
309 Supplementary Table 1). Altogether, our data indicate that reduction of *Erb4* may alter the metabolic
310 functions in female mice.

311

312 **Reduction of *Erb4* signalling enhances production of intrafollicular and circulating *Amh***

313 Women with PCOS have elevated circulating and intrafollicular AMH levels related to an
314 increase in the number of follicles and from hypersecretion by granulosa cells (37). We analysed *Amh*
315 mRNA expression by qPCR and *in situ* hybridization in the *Erb4^{Flox/Flox}; AmhCre⁺* and WT ovaries.
316 *Amh* expression was upregulated significantly ($P < 0.05$, Fig. 8A) in the *Erb4^{Flox/Flox} MisCre⁺* ovaries
317 compared to the WT, while its receptor, *Amhr* expression remained unchanged (Fig. 8A). *In situ*
318 hybridization on the ovaries of 3-month-old and 6-month-old mice revealed *Amh* expression in the
319 primary to the early antral follicles of both mice, but in the *Erb4^{Flox/Flox} AmhCre⁺* females the
320 abundance and number of follicles expressing *Amh* was higher than in the WT (Fig. 8B-E). To follow
321 up on these results, we then measured the circulating *Amh* levels. *Erb4^{Flox/Flox} AmhCre⁺* mice showed
322 higher serum *Amh* levels compared to the WT mice ($P < 0.05$ at two-three-months $n = 4$ for each
323 genotype, and $P < 0.001$ at six-eight-months $n = 4$ for each genotype, Fig. 8F).

324 To further investigate the molecular mechanism underlying the effect of the loss of *Erb4* in
325 our mouse model, we took advantage of KK1 mouse granulosa cell line (35) to analyse further how
326 *Erb4* signalling might affect *Amh* expression in granulosa cells. To achieve down-regulation of
327 *Erb4* in KK1 cell line, we used lentivirus (shRNAs) against mouse *Erb4*. *Erb4* full length protein
328 and ICD were produced in KK1 non-transfected granulosa cells and in transfected granulosa cells

329 with scramble shRNA and Erbb4sh2, whereas in transfected granulosa cells with sh1 and sh3 Erbb4
330 both full-length protein and ICD were downregulated. For further analysis, we will focus only Erbb4-
331 sh1 and -sh3 (Fig. 8G). Our data showed that Amh was up-regulated in both the ovary and in the
332 serum of *Erbb4^{Flox/Flox}; AmhCre⁺* mice (Fig. 8A, F), we further used the same set-up to analyse the
333 role of Erbb4 in the regulation of Amh. In addition, based on the results obtained from *in vivo*
334 granulosa cells (Fig. 1G), demonstrating that *Hb-Egf* mRNA was more expressed in granulosa cells
335 than in cumulus cell and since Hb-Egf is known to be one of the ligand for Erbb4 receptors, we then
336 examined the addition of Hb-Egf on the KK1 cell line. When Hb-Egf was added to the cell cultures,
337 *Amh* mRNA was up-regulated in KK1 cell line transfected by Erbb4-sh3 compared to the non-treated
338 ones ($p < 0.001$, Fig. 8H) suggesting the crosslink between Erbb4 and Amh via Hb-Egf. Collectively,
339 this data demonstrates that Erbb4 signalling regulates the expression of Amh in the granulosa cells
340 and its production into the serum.

341

342 Discussion

343

344 In human ovarian follicles, *ERBB4* is known to be expressed in mural and cumulus granulosa
345 cells (38). Several key traits of PCOS are on the reproductive, endocrine and metabolic characteristics
346 shown in different animal models but how PCOS phenotype stems is still unclear. In this current
347 study, we provide evidence that supports *Erb4* signalling as a player in the development of
348 experimental PCOS features (Table 1). Our previous study in which we analysed *Erb4* expression
349 by qPCR during embryonic ovarian development until birth had shown that *Erb4* is expressed at
350 low levels in embryonic ovaries (20). The current study reveals that *Erb4* expression is localised in
351 the granulosa cells of primary, secondary and antral follicles. We established a model lacking *Erb4*
352 gene in granulosa cells by crossbreeding *Erb4^{Flox/Flox}* (26) with *AmhCre* (27) mice. Expression of
353 cleavable *Erb4* isoforms JM-a CYT-1 and JM-a CYT-2 was activated in association with
354 folliculogenesis. In the conditional *Erb4* loss-of-function under the *Amh* promoter in ovaries, *Erb4*
355 isoform JM-a CYT-2 was reduced resulting in the activity loss of the membrane-anchored
356 intracellular domain (ICD), whereas JM-a CYT-1 isoform was less affected. ICD translocates to the
357 nucleus and regulates factors that activate or repress transcription (25). The observed *Erb4*
358 expression pattern suggests that this signalling protein has a role in follicle development and the
359 *Erb4^{Flox/Flox} AmhCre⁺* mice expressed several PCOS-like symptoms.

360 PCOS-like symptoms include infrequent or abnormal menstruation (5), increased body weight
361 (4), a number of antral follicles developing large-follicle cysts increased intra-ovarian and circulating
362 AMH concentrations and metabolic changes (3). In our studies, *Erb4^{Flox/Flox} ; AmhCre⁺* females
363 showed a disturbed oestrous and a reduced number of corpora lutea, suggesting non-regular ovulation.
364 Moreover, female total body weight was higher from P25 until the age of six months, associated with
365 bigger adipocyte size, and also hyperandrogenism was observed with thickness of theca cells layer in
366 the mutant mice. Our mouse model demonstrated an increase in the number of secondary stage

367 follicles in combination with increased *Amh* mRNA expression in the ovaries, as well as increased
368 circulating *Amh* levels starting at the age of 2 to 3 months until the age of 6 to 8 months. Using an *in*
369 *vitro* cell culture assay, we confirmed that a reduction of the expression of *Erb4* lead to the up-
370 regulation of *Amh* mRNA, a similar phenotype as in the conditional knock out mice. We strongly
371 suspect that all these characteristics in the *Erb4^{Flox/Flox}; AmhCre⁺* females are similar to those in
372 PCOS patients (37).

373 Beyond the morphological ovarian abnormalities, high levels of androgens and AMH in
374 PCOS patients are suspected to contribute to follicle growth arrest (4). Hyperandrogenism is
375 attributed to elevated circulating LH levels and acquisition of *Lhcgr* by the follicles during early
376 stages of development (3). Growing follicles, on the other hand, secrete AMH locally, arresting their
377 progression. This process is associated with a decreased sensitivity of the granulosa cells to FSH (4).
378 In *Erb4^{Flox/Flox}; AmhCre⁺* mice, elevated LH levels increased *Lhcgr* in growing follicles and lipid
379 content in ovarian theca cells were associated with an upregulation of *Hsd3b1* and *Hsd17b1*. The
380 expression of both enzymes results in increased androstenedione and testosterone level. As an
381 elevated circulating *Amh* concentration and mRNA content in the ovary are observed, all these results
382 suggest a similar endocrine condition as in PCOS patients (39,40).

383 Tata *et al.* (12) mouse model develops PCOS-like traits due to the exposure to *Amh* excess
384 during pregnancy impacting brains, ovaries and placenta of the maternal and foetal females leading
385 to gestational hyperandrogenism with hyper-LH driving androgen hypersecretion. By comparing our
386 developed model, we may hypothesise that our mouse model might be similar to Tata's mouse model
387 where the female offspring might be exposed *in utero* to an excess of *Amh* and hyperandrogenism.
388 Therefore, such study needs to be carried out to portray the importance of *Erb4* signalling in the
389 establishment of PCOS-like traits.

390 The *Erb4^{Flox/Flox}; AmhCre⁺* follicles presented a range of phenotypes including entrapped
391 oocytes as early as 25 days-old which was reinforced by ultrastructure defects between granulosa

392 cells and oocytes (reduced TZP, microvilli and *Gja1* expression). *KitL* is a critical factor for the
393 growth of oocytes, and its expression level is differentially controlled by paracrine and hormonal
394 factors. Gap junctional communications coupled granulosa cells via *Gja1* are necessary to respond to
395 *Gdnf9* and sustain *KitL* expression, however *Gdf9* and *Bmp15* influence the expression and the
396 relative abundance of *KitL* during folliculogenesis (41). *Gdnf9* and *KitL* expressions were up-
397 regulated in *Erb4^{Flox/Flox}; AmhCre⁺* ovaries and the oocyte phenotype particularities are coinciding
398 with a substantial reduction of TZP and microvilli in the *Erb4^{Flox/Flox}; AmhCre⁺* females.

399 TZPs are the extensions of the granulosa/ cumulus cell membranes that provide direct contact
400 with the other granulosa cells and in response oocyte membrane projects microvilli by forming
401 adhesive and gap junctions (33). Through the gap junctions nucleic acids, proteins (42) and even
402 lipids can be transported (43). In addition, the interplay between this cargo transport and, particularly,
403 paracrine growth factors and hormones is hypothesized to be involved in the selection, support and
404 atresia of the follicles. It appears that the observed follicle phenotypes could be the result of the
405 shortened connections, but why a lack of *Erb4* affects these junctions is unclear. During the oocyte
406 maturation phases, the number of TZPs and the proportion of adherent junctions to gap junctions
407 fluctuates. At the final stage of oocyte maturation, the TZPs are shorter than at the beginning of the
408 maturation process. This retraction is attributed to a surge of LH (44), so it is possible that TPZ
409 shortening is an indirect effect of *Erb4* knockout through elevated LH levels. Physiologically,
410 cumulus cells are not target cells for LH action since they barely express detectable levels of *Lhcgr*.
411 As a result, involvement of paracrine factors of the mural granulosa cells in the form of epidermal
412 growth factor (EGF) -like peptides must be required. These soluble EGFs in turn function in both an
413 autocrine and paracrine fashion to activate *Erb4* on theca and granulosa cells. From these EGF-like
414 factors Hb-EGF and Ereg which can bind to both *Erb4* and *Erb1*, activate a downstream *Erb4*
415 signalling pathway (24). When the *Erb4* pathway is activated and functional in the mural granulosa
416 cells, the *Erb4* signal is transmitted to the cumulus cells and the oocytes, and plays an essential role

417 during oocyte maturation, cumulus expansion, ovulation and luteinisation of granulosa cells to sustain
418 any pregnancy (45,46). Therefore, mice without functional *ErbB1* in the granulosa cells show an
419 inhibition of oocyte maturation *in vivo* and defective ovulation (45). We could hypothesise that *ErbB4*
420 and *ErbB1* could work either together or separately and have different signalling pathway
421 characteristics.

422 Hb-Egf interacts with either *ErbB1* or *ErbB4* receptor. Iwamoto *et al.* shows that Hb-Egf
423 binding to *ErbB4*-isoform JM-a CYT-2 results to the cleavage of ICD which inhibits the valve
424 mesenchymal cell proliferation whereas Hb-Egf binding to *ErbB1* induces cell proliferation in the
425 valves, during heart development (47). This example demonstrates that Hb-Egf might selectively bind
426 to *ErbB* receptors. We observed that the addition of Hb-Egf in KK1 granulosa cells transfected with
427 sh1 or sh3 against *ErbB4* did not rescue *Amh* expression. Hb-Egf needs an interaction with heparan
428 sulfate proteoglycans (HSPGs) through the heparin-binding domain of Hb-Egf. During remodelling
429 of female reproductive organs HSPG is reported to recruit Hb-Egf and *ErbB4* (48). Combining our
430 results and the literature, we believe that Hb-Egf recruits *ErbB4* either as a homodimer *ErbB4:ErbB4*
431 or as an heterodimer *ErbB1:ErbB4* in granulosa cells for the follicle development to prepare the
432 oocytes to be ovulated and initiated the luteinisation of granulosa cells.

433 The PCOS phenotype also includes metabolic risks such as insulin resistance, abdominal
434 obesity and high cholesterol (10). Androgen excess is associated with metabolic risk in PCOS, and
435 patients can be divided into two categories depending on their weight: the obese and lean. Obese
436 women with hyperandrogenism are often hyperinsulinemic, whereas lean women and
437 hyperandrogenism might have normal or hyper-insulin secretion and sensitivity (5). Obese and lean
438 PCOS women present alteration of intrafollicular paracrine signalling, and perturbations of the
439 processes of follicles activation, survival, growth, and selection (49). Insulin sensitivity varies by
440 PCOS phenotype and insulin resistance is not observed in all PCOS lean and obese women (50).
441 PCOS women, who have milder metabolic dysfunction or have a normal metabolism, might present

442 no hyperandrogenism phenotype (51). In our studies, we found that the *ErbB4*^{Flox/Flox}; *AmhCre*⁺ mice
443 basal non-fasting insulin level was induced, slight body and ovary weight was increased associated
444 with high circulating LH and lower androstenedione. We could further hypothesise that the observed
445 results in our mouse model might be reinforced by the chronic hypoestrogenism which will affect the
446 reduced estrous cycle frequency. If a lack of estradiol production or action is observed, such
447 consequence will be noticed on the weight and metabolism leading to weight gain and insulin
448 resistance. Combining all our results, we suggest that the *ErbB4*^{Flox/Flox}; *AmhCre*⁺ female mice present
449 a similar phenotype as the PCOS lean women phenotype.

450 In conclusion, our results indicate that disruption of the *ErbB4* gene by AmhCre recombinase
451 in female mice induces PCOS-like phenotypes. Our work provides evidence that confirms the GWAS
452 studies of European and Chinese cohorts that *ErbB4* contributes to oocyte developmental competence,
453 which is crucial for the proper development of an embryo.

454 **Materials and Methods**

455

456 **Animals**

457 *ErbB4^{Flox/Flox}* (26), Rosa26LacZCre (52) and *AmhCre* (27) mice were used. To generate *ErbB4^{Flox/Flox}*
458 ; *AmhCre⁺* mice, *ErbB4^{Flox/-} AmhCre⁺* heterozygote males were mated with *ErbB4^{Flox/Flox}* females.
459 The gonads of the wild-type littermate (WT) embryos were used as controls. All the experiments
460 involving mice were in accordance with the Finnish national legislation, the European Convention
461 (ETS 123), and EU Directive 86/609/EEC. Samples were collected and genotyped by PCR as
462 previously described (26,27,52). Positive WT and *ErbB4^{Flox/Flox}* ; *AmhCre⁺* samples were fixed in 4%
463 paraformaldehyde (PFA) overnight at +4°C, washed in 0.1 M potassium phosphate buffer (PBS) at
464 room temperature for 1 h, graded into 25%, 50% and 75% ethanol in PBS for 2 hours and stored at -
465 20°C until processing for immunohistochemistry and *in situ* hybridization as previously described
466 (20).

467

468 **Fertility assay, oestrous cycle assessment, histology and hormone level determination**

469 To test fertility, single-pair matings were done between 2-month-old *ErbB4^{Flox/Flox}* ; *AmhCre⁺* females
470 or WT littermates and 3-month-old *ErbB4^{Flox/Flox}* males. Ten matings were done for each genotype.
471 To determine the oestrous cycle stage of the female mice, a cytological analysis of the vaginal smears
472 was performed for 20 consecutive days using 7 mice for each genotype. Briefly, in the morning, the
473 vagina of the mouse was aspirated with a drop of sterile PBS and the secretion was smeared on an
474 objective glass. The smears were then air dried, fixed with ethanol, stained with Mayer hematoxylin
475 (Sigma-Aldrich, St Louis, MO, USA) and washed in tap water. The stage of the oestrous cycle was
476 determined by analysing the cell number and type under a microscope (53).
477 Histology was proceed as described previously (17). The quantification of ovarian follicles was
478 performed from consecutive histologic sections of a thickness of 7 µm from seven *ErbB4^{Flox/Flox}* ;

479 *AmhCre*⁺ and three WT mice. The morphological classification of follicles was carried out according
480 to previously described staging (54), and each histologic section was compared with previous and
481 sequential sections to prevent counting the same follicles more than once.

482 Blood from *ErbB4*^{Flox/Flox}; *AmhCre*⁺ and WT littermate females was collected at the age of two, three,
483 six and eight months. The serum was separated by centrifugation and stored at -80°C until further
484 analysis. FSH and LH levels were measured by immunofluorometric assays as previously described
485 (55). Androstenedione, progesterone, estradiol (E2) and testosterone were analysed in a single run by
486 a validated gas chromatography tandem mass spectrometry method (30). Serum insulin levels were
487 determined by insulin Rodent (Mouse/Rat) Chemiluminescence ELISA kit (ALPCO, 80-INSMR-
488 CH01, USA), according to the manufacturer's instructions. Serum Amh levels were detected using a
489 mouse Amh competitive ELISA kit (CUSABIO; #CSB-E13156m, USA), according to the
490 manufacturer's protocol. Serum leptin levels were detected using a mouse/rat leptin competitive
491 ELISA kit (ALPCO, 22-LEPMS-E01, USA), according to the manufacturer's protocol. A minimum
492 of five serum for each stage and genotype were analysed with the selected immunoassay.

493

494 **β-galactosidase staining**

495 For β-galactosidase staining, Rosa26LacZCre ovarian tissues were immersed in a 15% and 30%
496 sucrose solution for 15 min each, embedded in cryoMount (Leica Biosystems, Wetzlar, Germany),
497 cut into 6 μm thick sections and stored at -80°C. Sections were air dried for 15 minutes, rinsed once
498 in PBS and fixed in 0.2% glutaraldehyde, 2mMMgCl₂, 5mMEGTA, and 0.1 M potassium phosphate
499 buffer at room temperature for 1 hour. After fixation, the tissues were washed with agitation in
500 2mMMgCl₂, 5 mM EGTA, 0.01% Na deoxycholate (Sigma-Aldrich, St. Louis, MO, USA), 0.02%
501 NP-40 (Roche Applied Sciences, Indianapolis, IN, USA) and 0.01 M potassium phosphate buffer.
502 The tissues were stained overnight at room temperature with 10 mM K₄Fe (CN)₆, 10 mM K₃Fe (CN)₆,
503 and 1 mg/ml X-gal (Fisher Scientific, Waltham, MA, USA). After staining, the tissues were washed,

504 post-fixed with 4% PFA, mounted with Shandon™ Immu-Mount (Fisher Scientific, New Hampshire,
505 USA) and photographed. The experiments were carried out on 4-5 mice per group.

506

507 **Immunohistochemistry**

508 Paraffin embedded ovaries were cut into 6 µm thick sections. Before any staining, the paraffin
509 sections were immersed in xylene (Sigma Pharmaceuticals, St Louis, MO, USA), washed twice in
510 absolute ethanol and rehydrated. For histological analysis, the sections were stained with hematoxylin
511 and eosin or with periodic acid–Schiff. For immunohistochemical analysis, antigen retrieval was
512 performed in a pressure cooker for 20min in 10 mmol/L citrate buffer (pH 6.0). The sections were
513 then washed in PBS with 0.05% Tween (PBS-T), blocked with 10% goat serum (Vector Laboratories,
514 Burlingame, CA, USA) solution in PBS for 30 min at room temperature in a humidifying chamber.
515 After blocking, primary antibodies against goat anti mouse Erbb4 (Errb4: sc-283, Santa Cruz), rabbit
516 anti-mouse Connexin 43/Cnx43 (71–0700; Life Technologies, Carlsbad, CA, USA), rabbit anti-
517 mouse Collagen IV (AB756P; Merk-Millipore, Burlington, MA, USA) and rabbit anti-mouse β-
518 Catenin/Ctnb-1 (no. 9561; Cell Signaling Technologies, MA, USA) diluted in 1% goat serum solution
519 in PBS-T were applied and incubated in a humidifying chamber at 4°C overnight. Samples were
520 washed in PBS-T three times for 10 minutes and secondary antibodies conjugated with Alexa Fluor
521 488- or Alexa Fluor 546 (Invitrogen, Life Technologies, Paisley, United Kingdom) were added and
522 incubated for one hour at room temperature. Sections were counterstained with DAPI for 10 minutes
523 at room temperature (Sigma-Aldrich, St. Louis, MO, USA). The samples were washed again and
524 mounted in Shandon™ Immu-Mount (Fisher Scientific, NH, USA). Erbb4 immunohistochemistry
525 was carried out, as previously described (17) except that sc-283 (1:50) (Santa Cruz Biotechnology,
526 Santa Cruz, CA, USA) was used as the primary antibody. The slides were photographed using a
527 confocal microscope (Olympus Fluoview FV10-ASW, Tokyo, Japan). All images were taken

precisely with the same parameters for both groups. A minimum of three ovarian sections for each stage and genotype were examined with selected antibodies.

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from collected granulosa cells or oocytes of one-month-old mouse, picked up by mouth pipetting and granulosa cells from super-ovulated mice using Trizol method.

The total RNA was isolated from the snap frozen tissues of 1-month-old WT mice using the RNeasy kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions and dissolved in RNase-free water. The concentration of the isolated RNA was determined using Nanodrop (Fisher Scientific, USA). The RNA samples were DNase treated with DNase I Amplification Grade Kit (Invitrogen, Thermo Fisher Scientific, USA). 100 ng of the total RNA was converted into cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) and used for real-time reverse transcription reactions.

To obtain RNA from the ovaries of 2 and 3-month-old *ErbB4^{Flox/Flox}* ; *AmhCre⁺* and WT littermate female mice, the total RNA from sectioned paraffin ovaries was extracted using RecoverAll Total Nucleic Acid Isolation Kit (Fisher Scientific, NA, USA). The purified RNA (0.2 µg) was converted into cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Each qPCR reaction consisted of 2 µl of cDNA, 2.5 µM of the primers, and 5 µl of Brilliant III SYBER Green mix (Agilent Technologies) in a total volume of 10 µl. The following primers were used: *Amh*, *Amhr2*, *Fst*, *Nobox*, *Gdnf9*, *Kit*, *Kitl*, *Gapdh* (36), *Foxl2* (56), *Cre* and *ErbB4* total and isoforms (*Jma*, *Jmb*, *Cyt-1* and *Cyt-2*) (17) and *Ppia* (57). The qPCR program consisted of 40 cycles at 95°C for 30 seconds and at 60°C for 1-minute run in Bio-Rad CFX96 thermocycler (Bio Rad, CA, USA). Housekeeping genes *Ppia*, *Gapdh* and *β-actin* were used for normalizing the data using the $\Delta\Delta CT$ method.

553 ***In situ* hybridization**

554 The nonradioactive *in situ* hybridization analysis of *ErbB4*, *Hsd3b1*, *Hsd17b1*, *Cyp19a1* and *Amh*
555 expression was carried out as described earlier (20). The probes for *ErbB4*, *Hsd3b1*, *Hsd17b1*,
556 *Cyp19a1* and *Amh* have been described previously (36,58). A minimum of three ovary-derived
557 sections for each genotype and stage were examined for the selected genes.

558 To detect *Lhcgr* transcripts we used RNAscope® 2.5 HD Reagent Kit-BROWN (#322300, Advanced
559 Cell Diagnostics, CA, USA) (59). The probes for *Lhcgr* (#408171, Advanced Cell Diagnostics, CA,
560 USA), *Ppib* (positive control reference probe, #313911, Advanced Cell Diagnostics, CA, USA) and
561 dapB (from *Bacillus S.*, nonsense probe, #310043, Advanced Cell Diagnostics, CA, USA) were
562 hybridized accordingly to the manufacturer's protocol. Images were taken using Leica DM4 B
563 microscope (Leica, Wetzlar, Germany.)

564

565 **Transmission electron microscopy (TEM)**

566 The dissected ovaries of 3-month-old mice (n = 3 for each genotype) were fixed in 1% glutaraldehyde
567 and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, post-fixed in 1% osmium tetroxide,
568 dehydrated in acetone, and embedded in Epon LX 112 (Ladd Research Industries, USA). The semi-
569 thin sections (1 µm) were first stained with toluidine blue for light microscopic inspection. Next, the
570 thin sections (80 nm) were cut with a Leica Ultra cut UCT ultra microtome, stained in uranyl acetate
571 and lead citrate, and examined in a Tecnai Spirit G2 transmission electron microscope (FEI Company,
572 USA). Images were captured with a Veleta CCD camera (Olympus Soft Imaging Solutions GMBH,
573 Munster, Germany).

574

575 **Western Blot**

576 The ovaries were prepared from 3-months-old mice. The proteins were purified immediately and
577 subjected to Western blotting using anti-ErbB4 (E200; Abcam), anti- α tubulin (T5168; Sigma)

antibodies as described previously (60). The bands were quantified using Image J software. The stable virus transfected cell lines were lysed using the lysis buffer as described previously (60).

Measurement of Granulosa cell, Theca cell and adipocyte size

For the measurement of granulosa cell space and theca cells, signal quantification was performed using Image J software 1.52p; to measure the threshold auto-threshold was integrated to enhance the contrast between white and black. Then set measurement was adjusted for the area minus the corrected threshold.

Four mice per group were randomly selected, and fixed gonadal adipose tissue was processed and analyzed. In brief, adipose depots were embedded in paraffin and, after routine histological preparation, sectioned at 6 μ m. Mounted sections were stained with hematoxylin and eosin. The diameter of adipocytes was measured using ImageJ software.

shRNA and cell culture

KK1 cells were grown in DMEM with 10% FBS (Sigma) and 1% antibiotics. The cells were transfected with short hairpin RNA (shRNA) against mouse Erbb4 (sh41 NM_010154 TRCN0000360692; sh42 NM_010154 TRCN0000360891; sh43 NM_010154 TRCN0000360689 and scramble shRNA). The transfected cells were selected using puromycin and stable cell lines were generated for further analyses. Cultures were supplemented with Hb-Egf (10 ng/ml, R&D System). Cell pellets were stored for either protein or RNA extraction as described above.

Statistical analysis

For comparisons between 2 groups, statistical significance was determined using the 2-tailed Student's *t* test and for all quantitative comparisons, 2-way ANOVA with Bonferroni's multiple comparisons test was performed with GraphPad Prism 7.03 software. Values of $P \leq 0.05$ and lower

603 were considered statistically significant and the results were presented as mean standard deviation
604 (mean $\pm$ SD). Asterisks indicate statistical significance according to this legend: * $P < 0.05$; ** $P < 0.01$;
605 *** $P < 0.001$.

606

607

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609

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617

618 **Conflict of Interest Statement**

619 None declared.

620

621 **References**

622

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793

794

795 **Figure legends:**

796

797 **Figure 1: Erbb4 expression in the ovary.** A) Alternative splicing generates four Erbb4 isoforms
798 that differ at the extracellular JM or the CYT domains. Tumour necrosis factor- α -converting enzyme
799 (TACE) and γ -secretase cleave JM-a isoforms to produce a soluble intracellular domain (ICD)
800 capable of translocating to the nucleus and regulating transcription. CYT-2 isoforms differ from
801 CYT-1 isoforms by lacking 16 amino acids known to include a PI3-K binding site and a proline-rich
802 interaction motif for WW domain-containing proteins. B) Expression of the *Erbb4* mRNA in WT
803 granulosa cells (GC), super ovulated GCs (hCG) and oocytes. C) Expression of transcripts encoding
804 all four *Erbb4* isoforms was analysed at 2 months-old ovaries by real-time qPCR. *Ppia* was used as
805 a reference gene to normalize expression levels. D-E) *In situ* hybridization analysis of *Erbb4* in 2
806 months old mouse ovaries demonstrated expression in the granulosa cells. F) Immunohistochemical
807 analysis of ErbB4 expression in 17 days old mouse ovary demonstrated expression in the granulosa
808 cells from primary to pre-antral follicles consistent with the *in situ* hybridization analysis. Higher
809 magnification of antral follicle stained with an antibody (sc-283) recognizing the carboxy-terminus
810 of ErbB4 demonstrated nuclear immunoreactivity in granulosa cells (black arrows). G) Expression of
811 transcripts encoding all ligands binding to Erbb4 was analysed in *in vivo* and super-ovulated
812 granulosa cells by real-time qPCR (n=3). *Ppia* was used as a reference gene to normalize expression
813 levels. SF secondary follicle, AF antral follicle. Scale bar, (D, E) 500 μ m, (F) 50 μ m. Bars represent
814 means $\pm$ SD, 2-way Anova * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

815

816 **Figure 2: Conditional knockout of *Erbb4* in the granulosa cells**

817 A) Expression of *Erbb4* transcript was analysed at 2 months-old pituitary, uterus and ovaries by real-
818 time qPCR. B-G) Immunohistochemistry of Erbb4 protein was localised in the granulosa cells from
819 primary to pre-antral follicles in wild type (WT) at (B-D) and the expression was reduced in

820 *Erb4^{Flox/Flox};AmhCre⁺* granulosa cell (E-G). Black arrows depict the expression of *Erb4* in the
821 nucleus; H, I) Efficiency of *AmhCre*-mediated inactivation of the floxed *Erb4* allele by real-time
822 qPCR of 2-month-old *Erb4^{Flox/Flox};AmhCre⁺* (n=4, black column) and WT ovaries (n=4, white
823 column) by quantifying the isoform *JM-a*, *CYT-1* and *CYT-2*. J) Western blot analyses of *Erb4*
824 protein expression in the 3 months-old ovaries with *Erb4* floxed (F/F) targeted after *Mis-Cre*-mediated
825 recombination (*Erb4^{Flox/Flox};AmhCre⁺*) as compared to littermates lacking *Erb4* transgene
826 (*Erb4^{Flox/Flox};AmhCre⁻*). The membranes were reblotted with actin and *ErbB4* to control for loading
827 and total *ErbB4* protein levels, respectively. A mean reduction of 1-fold was observed in total *Erb4*
828 protein. Columns represent total *Erb4* Western levels (expression of full-length 180 kD receptor plus
829 the 80 kD processed fragment, intracellular domain ICD) quantified by densitometry. Scale bar, 20
830 μ m (B-G). Bars represent means $\pm$ SD, 2-tailed Student's t-test * $P \leq 0.05$, *** $P \leq 0.001$.

831

832 **Figure 3: Ovulation is impaired in *Erb4^{Flox/Flox};AmhCre⁺* mice affecting fertility.**

833 A) Number of progenies per mouse (n = 10 females from each genotype were mated). B–D) Oestrous
834 cycle of females aged two, three and five months *Erb4^{Flox/Flox};AmhCre⁺* (n=7, black column) and
835 WT (n=7, white column) ovaries. E–J) Histological sections of 35 days, 3-month-old and 6-month-
836 old WT and *Erb4^{Flox/Flox};AmhCre⁺* ovaries. K) Follicle counts at 35 days and L) at 3 months in
837 *Erb4^{Flox/Flox};AmhCre⁺* mice (n=7, black column) and WT (n=3, white column). Number of corpus
838 luteum (CL) and atretic follicles (as depicted in Fig. 3E-J) in WT and *Erb4^{Flox/Flox};AmhCre⁺* mice.
839 Scale bar, 500 μ m (E-J). Bars represent means $\pm$ SD, 2-way Anova * $P \leq 0.05$, ** $P \leq 0.05$, *** $P \leq$
840 0.001.

841

842 **Figure 4: Oocyte failed to ovulate in *Erb4^{Flox/Flox};AmhCre⁺* secondary follicles.** A-J) Higher
843 magnification of WT oocytes (A, D). Higher magnifications showing oocyte defects that have
844 condensed cytoplasmic fragmentation (B, H), chromatin (E) or are undergoing breaking (C, F-H).

atretic oocytes (I) and entrapped oocyte in granulosa luteinised cells (J) were observed. K) Number of oocytes classified as abnormal in E to I compared to normal oocytes (*Erb4^{Flox/Flox};AmhCre⁺* n=7, black column) and WT (n=3, white column) in ovarian cross-sections. L) Expression of transcripts encoding oocyte (*Gdnf9*, *Nobox*, *c-Kit*, *Sohlh1*) and granulosa cell markers (*Fst*, *Kitl*) was analysed in *Erb4^{Flox/Flox};MisCre⁺* mice (n=5) and WT (n=5) 3 months-old ovaries by real-time qPCR. *Ppia* was used as a reference gene to normalize expression levels. Scale bar, 20 μ m (A-I) and 100 μ m (J). Bars represent means $\pm$ SD, 2-tailed Student's t-test * $P < 0.05$, ** $P < 0.01$.

Figure 5: Hyperandrogenism and LH hypersecretion are observed in *Erb4^{Flox/Flox};AmhCre⁺* mice. A-D) Hormone levels of androstenedione, progesterone, oestradiol and testosterone measured during the oestrous cycle (protrous (P); estrous (E) and metestrous (M)) at the age of three months. E, F) LH and FSH measured at diestrus (D) for 2 and 3-month-old and 6-month-old females. G, H) *Lhcgr* RNAscope *in situ* hybridization of 3-month-old WT and *Erb4^{Flox/Flox};AmhCre⁺* ovaries at diestrus stage. I-P) *Hsd3b1*, *Hsd17b3*, *Cyp19a1* *in situ* hybridization (brown colour) of 3-month-old WT and *Erb4^{Flox/Flox};AmhCre⁺* ovaries. Arrows show the theca cells whereas the yellow arrowheads show granulosa cells. PF primary follicle, SF secondary follicle, PA pre-antral follicle, CL corpus luteum. Scale bar 500 μ m (I, J, M-P), 100 μ m (G, H, K, L). Bars represent means $\pm$ SD, 2-tailed Student's t-test * $P < 0.05$, ** $P < 0.01$

Figure 6: Thickness of theca cells layer in *Erb4^{Flox/Flox};AmhCre⁺* mice. A-F) Electron microscopy images of the WT (n=3) and *Erb4^{Flox/Flox};AmhCre⁺* (n=3) ovaries of 3-month-old mice. (C-F) Higher magnification of granulosa and theca cells. G) Quantification of the granulosa cell space. H) Quantification of theca cells layer around the follicles. I, J) Higher magnification of the transzonal processes (TZPs, red arrows) between granulosa cells and microvilli between granulosa cells and oocyte (black arrows) from the same follicles shown in A and B. GC granulosa cell, Oo oocyte, TC

870 theca cell. Scale bars correspond to 100 μm (A, B); 10 μm (E-H), 2 μm (C, D). 2-tailed Student's t-
871 test $*P \leq 0.05$.

872

873 **Figure 7: Obesity and metabolic changes are observed in *ErbB4^{Flox/Flox};AmhCre⁺* mice.** A-C)
874 Weights of WT and *ErbB4^{Flox/Flox};AmhCre⁺* body and ovaries, C) ratio of ovary weight on total body
875 weight (*ErbB4^{Flox/Flox};AmhCre⁺*, NB n=3, P10 n=10, P25 n=11, 6 weeks n=13, 3 months n=33, 6
876 months n=36, black column) and WT ovaries (NB n=5, P10 n=5, P25 n=9, 6 weeks n=5, 3 months
877 n=30, 6 months n=31 white column). NB new-born, P10 post embryonic day 10, P25 post embryonic
878 day 25; D) Insulin level from non-fasting WT and *ErbB4^{Flox/Flox};AmhCre⁺* mice. E, F) Haematoxylin
879 and eosin staining of section of gonadal adipose depots in WT (n=3) (A) and *ErbB4^{Flox/Flox}; AmhCre⁺*
880 (n=3) at three-months-old mice. G) Adipocyte diameter in *ErbB4^{Flox/Flox}; AmhCre⁺* mice (n=3) and
881 WT (n=3) mice. Scale bar, 100 μm . Bars represent means $\pm$ SD, 2-way Anova for A and B, 2-tailed
882 Student's t-test C and F $*P \leq 0.05$.

883

884 **Figure 8: *Amh* is upregulated in ovaries and serum from *ErbB4^{Flox/Flox};AmhCre⁺* mice.** A) mRNA
885 expression of the *Amh* and *Amhr2* in 3-months-old WT and *ErbB4^{Flox/Flox};AmhCre⁺* ovaries. *Ppia* was
886 used as a reference gene to normalize expression levels. B-E) *Amh* *in situ* hybridization (brown
887 colour) of 3-month-old and 6-month-old WT and *ErbB4^{Flox/Flox}; AmhCre⁺* ovaries. F) Circulating *Amh*
888 in 3-, 6-8-months-old WT and *ErbB4^{Flox/Flox};AmhCre⁺* mice. G, H) Western blot analyses of *ErbB4*
889 protein expression in transfected KK1 cell line. Sh1-3 are the shRNA against *ErbB4* and scr scramble
890 shRNA. The membranes were reblotted with alpha-tubulin and *ErbB4* to control for loading and total
891 *ErbB4* protein levels, respectively. Columns represent total *ErbB4*. Western levels (expression of full-
892 length 180 kD receptor plus the 80 kD processed fragment, intracellular domain ICD) quantified by
893 densitometry. I) Expression of transcripts encoding *Amh* was analysed by real-time qPCR in *in vitro*
894 KK1 cell line transfected with sh1-sh3 and with or without Hb-Egf. *Ppia* was used as a reference

895 gene to normalize expression levels. Scale bars correspond to 500 μm (B-F), Bars represent means $\pm$
896 SD, 2-tailed Student's t-test * $P \leq 0.05$.

897

898 **Graphical abstract: Proposed model for the mechanism by which Erbb4 may influences**
899 **folliculogenesis and fertility.** In healthy women, Errb4 receptor is expressed in granulosa cells,
900 following its binding to Hb-Egf. The ICD is then translocated to the nucleus. ICD binds to the DNA
901 to enhance or inhibit the expression of numerous genes (*Gja1*, *Fst*, *Amh* and *Kit-L*). Thus, the gene
902 activation or inhibition results in the strong adhesion between granulosa cells and decreased level of
903 AMH that are required for normal follicles development. Consequently, the microenvironment and
904 the genes expression are favourable for the normal folliculogenesis. In PCOS condition, Errb4 is
905 significantly downregulated resulting in non-translocated ICD signalling. As well, LH hypersecretion
906 and hyperinsulinemia in the blood reinforce the dysfunction of the granulosa cells. As consequences,
907 *Gja1* expression level is substantially decreased leading to disruption of the adhesion between the
908 granulosa cells as well as significant increase of *Amh* hormone. Based on the un-welcoming
909 microenvironment, the development of the follicles is not occurring properly and thus PCOS features
910 are taking place.

911

912

913 **Table 1:** Summary of the effect of *Erb4* deletion on the development of PCOS traits.

914

915	Clinical PCOS trait*	<i>Erb4</i> deletion (<i>Erb4</i> ^{Flox/Flox} <i>AmhCre</i> ⁺)
916	Absent/irregular cycling	√
917	Oligo/anovulation	partial
918	Multicystic ovary	~
919	↑Change in ovary weight	√
920	↓Granulosa cell layer thickness	~
921	↑Theca cell-layer thickness	√
922	↑Body weight	√
923	Adipocyte hypertrophy	√
924	↑Leptin	~
925	↑Insulin	√

926

927 *in human

928 √ clinical PCOS trait present

929 ~ clinical PCOS trait not present

930

931 **Abbreviation**

932	Amh	anti-Müllerian hormone
933	Bmp15	Bone morphogenetic protein 15
934	Btc	betacellulin
935	CL	corpus luteum
936	Cyp19a1	Cytochrome P450 Family 19 Subfamily A Member 1
937	CYT	intracellular cytoplasmic domain
938	EGF	epidermal growth factor
939	ErbB4	receptor tyrosine kinase 4
940	Ereg	Epiregulin
941	FSH	follicle stimulating hormone
942	GC	granulosa cell
943	Gdnf9	growth differentiation factor 9
944	Gja1	Gap Junction Protein Alpha 1
945	GWAS	genome wide association studies
946	Hb-EGF	Heparin-binding EGF-like growth factor
947	hCG	super ovulated granulosa cells
948	Hsd3b1	3beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase type I
949	HSPG	heparan sulfate proteoglycans
950	ICD	intra-cellular domain
951	JM	extracellular juxtamembrane
952	KK1	KK1 cell line
953	KitL	kit ligand
954	LH	luteinizing hormone
955	Lhcgr	luteinizing hormone/choriogonadotropin receptor

956 mRNA messenger ribonucleic acid

957 Nrg neuregulin

958 oo oocyte

959 P35 post-natal 35 days

960 PA pre-antral follicle

961 PCOS Polycystic ovary syndrome

962 PF primary follicle

963 qPCR quantitative polymerase chain reaction

964 SD standard deviation

965 SF secondary follicle

966 scr scramble

967 shRNA short hairpin ribonucleic acid

968 TZP transzonal projections

969 WT wild type

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

***Erb4* regulates the oocyte microenvironment during folliculogenesis**

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Supplementary figure 1: Positive LacZ expression in granulosa cells of *Rosa26LacZAmhCre*⁺ in 3-month-old ovaries. A) highlighted oocyte (oo) was negative.

Supplementary figure 2: Pituitary and uterus of *Erb4*^{Flox/Flox};*AmhCre*⁺ mice. A) Pituitary, B) uterus weight. C, D) Haematoxylin and eosin staining of uterus. Scale bars correspond to 100 µm.

Supplementary figure 3: Reduced fecundity of *Erb4*^{Flox/Flox};*AmhCre*⁺ mice. A) Number of pups per litter from crosses with WT males (n = 10 females from each genotype were mated). 2-tailed Student's t-test **P* ≤ 0.001.

Supplementary figure 4: *Erb4* deficiency in the granulosa cells affects cell junction. A-F) Type IV collagen (Col4a1), connexin 43 (Gja1) and β-catenin (Ctnnb1) expression in follicles of 3-month-old WT and *Erb4*^{Flox/Flox};*AmhCre*⁺ ovaries. A, B) Arrows indicate the expression of Col4a1 in theca cells surrounding the secondary follicles. C, D) Arrows indicate the expression of the Gja1 in the antral follicle in WT compared to the mutant female where the protein is less abundant or misexpressed. In C and D, the insets show Gja1 expression alone in a follicle. E, F) Arrows indicate the expression of the Ctnnb1 in the secondary follicles in WT compared to the mutant female where the protein is strongly observed in the plasma membrane between the granulosa cells. GC granulosa cell, TC theca cell, Oo oocyte. Scale bars correspond to 100 µm.

Supplementary Table 1: Leptin serum level in female *Erb4*^{Flox/Flox};*AmhCre*⁺ and WT mice.

Supplementary figure 1

Supplementary Figure 2

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Supplementary figure 3

Supplementary Figure 4

Supplementary Table 1:

	WT	Erb4 ^{Flox/Flox} MisCre ⁺
2-3 months		
Leptin (ng/ml)	468 ± 391 (n=3)	342 ± 488 (n=4)
6-8 month		
Leptin (ng/ml)	294 ± 93 (n=4)	267 ± 167 (n=3)