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**Strap regulates early follicle development in the mouse ovary**

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1 **Serine threonine kinase receptor associated protein (Strap) regulates early**  
2 **follicle development in the mouse ovary**

3

4 **Short title:** Strap regulation of early follicle development

5

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16

17 **Key words:** TGFB, Strap, Smad, oocyte, granulosa cells, preantral, follicle  
18 development, follicle culture, ovary culture

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26 **Abstract:**

27

28 The molecular mechanisms involved in regulating the development of small,  
29 gonadotrophin-independent follicles are poorly understood; however, many studies  
30 have highlighted an essential role for TGFB ligands. Canonical TGFB signalling is  
31 dependent upon intracellular Smad proteins that regulate transcription. Strap has  
32 been identified in other tissues as an inhibitor of the TGFB-Smad signalling pathway.  
33 Therefore, in this study we aimed to determine the expression and role of Strap in the  
34 context of early follicle development. Using qPCR, *Strap*, *Smad3* and *Smad7*  
35 revealed similar expression profiles in immature ovaries from mice aged 4-16 days  
36 containing different populations of early growing follicles. Strap and Smad2/3 proteins  
37 co-localised in granulosa cells of small follicles using immunofluorescence. Using an  
38 established culture model, neonatal mouse ovary fragments with a high density of  
39 small non-growing follicles were used to examine the effects of *Strap* knock down  
40 using siRNA and Strap protein inhibition by immuno-neutralisation. Both interventions  
41 caused a reduction in the proportion of small, non-growing follicles and an increase in  
42 the proportion and size of growing follicles in comparison to untreated controls,  
43 suggesting inhibition of Strap facilitates follicle activation. Recombinant Strap protein  
44 had no effect on small, non-growing follicles, but increased the mean oocyte size of  
45 growing follicles in the neonatal ovary model and also promoted the growth of  
46 isolated preantral follicles *in vitro*. Overall findings indicate Strap is expressed in the  
47 mouse ovary and is capable of regulating development of small follicles in a stage-  
48 dependent manner.

49

50

51 **Introduction:**

52

53 The ovarian reserve consists of a limited number of small follicles each made up of  
54 an immature oocyte surrounded by a single layer of granulosa cells (GCs) (Hirshfield  
55 1991, Gougeon 1996). Throughout life, some of these follicles undergo  
56 developmental changes that precipitate an increased rate of oocyte growth and GC  
57 proliferation (Hirshfield 1991, Da Silva-Buttkus *et al.* 2008, Adhikari & Liu 2009). The  
58 molecular mechanisms that regulate this co-ordinated event are still unresolved;  
59 however, a number of ovarian-expressed growth factors and signalling molecules  
60 have been implicated, particularly in relation to the PI3 kinase and mTOR pathways  
61 (Adhikari & Liu 2009). The transforming growth factor beta (TGFB) pathway is also  
62 important in this context, since various mutations in specific ligands and receptors  
63 have revealed striking effects on early follicle development and consequentially,  
64 fertility (Dong *et al.* 1996, Galloway *et al.* 2000, Edson *et al.* 2009). **However, very  
65 little is known about the molecular regulation of TGFB signalling in small follicles, and  
66 how other factors interacting with this pathway may influence early growth or arrest.**

67

68 TGFB signalling involves ligand binding to specific Type-1 and Type-2 cell surface  
69 receptors where the kinase activity is propagated by phosphorylation of receptor-  
70 regulated Smad (R-Smad) proteins (Shi & Massague 2003). Activated R-Smads then  
71 form a complex with Smad4, which facilitates nuclear import and dwell time, leading  
72 to DNA binding and regulation of target genes (Wakefield & Hill 2013). Activins,  
73 TGFB1-3 and growth differentiation factor 9 (GDF9) tend to signal mainly via  
74 Smad2/3, while anti-Mullerian hormone (AMH) and bone morphogenetic proteins  
75 (BMPs) utilise Smad1/5/9 (Wakefield & Hill 2013). Transgenic mouse models  
76 harbouring GC-specific (conditional) mutations in each of these R-Smads have  
77 revealed fundamental roles for TGFB signalling in growing follicles (Li *et al.* 2008,  
78 Pangas *et al.* 2008, Middlebrook *et al.* 2009); however, the distinct role of this

79 pathway in small, single-layered (non-growing) follicles is still unclear. Recent studies  
80 have also highlighted distinct, differential expression of R-Smads in granulosa cells,  
81 with Smad1/5/8 localised in multi-layered, growing preantral follicles and Smad2/3  
82 predominantly localised in small, single-layered follicles (Xu *et al.* 2002, Fenwick *et*  
83 *al.* 2013). This indicates that Smad2/3 may be an important regulator of the very  
84 early stages of follicle development.

85

86 The TGFB pathway is highly conserved across a range of species, and within  
87 species is a pivotal regulator of numerous developmental processes that influence  
88 cell phenotype (Massague 2012, Wakefield & Hill 2013). Fine control of the TGFB  
89 pathway is achieved in part by the different interacting proteins (Wakefield & Hill  
90 2013). One example is Smad7, which is induced by R-Smads and acts to negatively  
91 regulate TGFB signalling by binding and promoting R-Smad or receptor degradation  
92 (Nakao *et al.* 1997, Itoh & ten Dijke 2007, Yan *et al.* 2016). Although the role of  
93 Smad7 during early follicle development has not been determined, recent studies  
94 have shown that Smad7 regulates TGFB signals and apoptosis of GCs from growing  
95 follicles (Quezada *et al.* 2012, Shimizu *et al.* 2012, Gao *et al.* 2013). In other cell  
96 types, the activity of Smad7, and consequently Smad2/3, is further regulated by  
97 serine threonine kinase receptor associated protein (Strap) (Datta *et al.* 1998). Strap  
98 acts by stabilising a complex between TGFBR1 and Smad7, thereby preventing  
99 Smad2 and Smad3 from access to the Type 1 receptor (Datta *et al.* 2000). Strap may  
100 therefore represent an indirect, negative regulator of TGFB signalling in the ovary.

101

102 In this study we aimed to determine if Strap plays a role in small follicles. We show  
103 that transcript levels of *Strap*, *Smad3* and *Smad7* exhibit similar expression profiles  
104 in immature mouse ovaries enriched with different proportions of non-growing and  
105 early growing follicles. Using an *in vitro* model to monitor follicle activation, we also  
106 show that Strap modulation using different interventions leads to changes in the

107 proportions of non-growing and growing follicles, consistent with a role for the TGFB  
108 pathway as an important regulator of early follicle development.  
109

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110 **Materials and Methods:**

111

112 *Animals and tissues*

113 All tissues used in this study were obtained from wild-type C57Bl6 mice housed  
114 under standard conditions in compliance with the Animals and Scientific Procedures  
115 Act, 1986, and also in accordance with approval from the University of Sheffield  
116 Training and Competency Officer. Ovaries were dissected from female mice at  
117 precisely 4, 8, 13 and 16 days of age (d4, d8, d13, d16) and finely dissected free of  
118 adhering tissue in isolation media consisting of Liebovitz L-15 (Life Technologies,  
119 ThermoFisher, Cheshire, UK) with 1% (w/v) bovine serum albumin (BSA; Sigma-  
120 Aldrich, Dorset, UK). Ovaries were used immediately for culture or were rinsed in  
121 PBS and frozen in liquid nitrogen for later RNA analysis or were fixed in 10% neutral  
122 buffered formalin (NBF; Sigma) for paraffin embedding, sectioning (5µm) and  
123 staining.

124

125 *RNA extraction, cDNA and quantitative PCR*

126 Total RNA was extracted from d4, d8 and d16 ovaries using RNeasy microcolumns,  
127 which includes a DNase digestion step (Qiagen; Crawley, West Sussex, UK). The  
128 quality of all RNA samples was assessed with an Agilent 2100 Bioanalyser (Agilent  
129 Technologies, Stockport, UK) and only those with an integrity value of 9-10 were  
130 included. An equivalent amount of RNA from all samples (50ng) was converted to  
131 cDNA using random hexamer primers and SuperScript III reverse transcriptase in  
132 accordance with the manufacturer's guidelines (Invitrogen, ThermoFisher, UK). For  
133 quantitative PCR assays, a reaction mixture consisting of nuclease-free H<sub>2</sub>O, Kapa  
134 SYBR Green and ROX dye (Kapa Biosystems Ltd., London, UK) was combined with  
135 500nM gene-specific primers (Table 1) and added to a 384-well plate. **All primers**  
136 **were initially confirmed for suitability for qPCR by the presence of a single band at**  
137 **the appropriate size by gel electrophoresis.** For each gene, an equal volume (1µl) of

138 cDNA or H<sub>2</sub>O (control) was added to each reaction in duplicate. An initial activation  
139 step at 95°C (3 minutes) was followed by cycling (40x) at 95°C (3 seconds), 58°C (20  
140 seconds) and 72°C (10 seconds) using an Applied Biosystems 7900HT Fast  
141 instrument (Applied Biosystems Inc., ThermoFisher, UK). Fluorescence was  
142 recorded at each cycle and also during a final DNA product melting protocol to  
143 ensure consistent and specific amplification. Final CT values were normalised  
144 against mouse *Atp5b* (PrimerDesign, Southampton, UK), which was stably  
145 expressed across all samples and used previously in similar experiments (Fenwick *et*  
146 *al.* 2011). Fold changes relative to d4 ovaries were calculated using  $2^{-\Delta\Delta CT}$  (Livak &  
147 Schmittgen 2001).

148

#### 149 *Immunohistochemistry*

150 Approximate midsections of d4, d8 and d16 ovaries were dewaxed in Histochoice  
151 solvent (Sigma) and re-hydrated in changes of ethanol of decreasing concentrations.  
152 Slides were immersed in 0.01M citrate buffer (pH6.0) and microwaved for 4 x 5  
153 minutes to retrieve antigens before washing in phosphate buffered saline (PBS;  
154 pH7.4). Non-specific binding was blocked with CAS-Block (ThermoFisher) for 20  
155 minutes before applying a mixture of rabbit anti-Smad2/3 (1:400; #5678; Cell  
156 Signalling Technology, Danvers MA) and mouse anti-Strap (0.3µg/ml; sc-136083;  
157 Santa Cruz Biotechnology, Dallas TX) diluted in CAS-Block overnight at 4°C. Primary  
158 antibodies were replaced on some sections with equivalent concentrations of non-  
159 immune mouse IgG (Vector Laboratories, Bakewell, UK) or rabbit IgG (Vector) to  
160 determine non-specific binding. After washes in PBS, all sections were incubated at  
161 room temperature for 45 minutes in a mixture of Alexa555 anti-mouse IgG (1:400;  
162 Invitrogen) and Alexa488 anti-rabbit IgG (1:400; Invitrogen) diluted in PBS. Sections  
163 were washed in PBS and mounted in ProLong Gold antifade reagent with DAPI  
164 (Invitrogen) and imaged using a Leica inverted SP5 confocal laser scanning  
165 microscope (Leica Microsystems, Wetzlar, Germany). Images presented in this study

166 were taken from sections stained in the same run, using the same laser and gain  
167 settings.

168

#### 169 *Ovary fragment culture*

170 Ovaries from d4 mice were finely cut into 6-8 equivalent sized pieces in drops of  
171 isolation media and placed into a single well of a 24-well cell culture plate (Sigma).  
172 Each well contained 1ml of culture medium consisting of MEM- $\alpha$  (ThermoFisher)  
173 supplemented with 10% (v/v) foetal bovine serum (FBS; ThermoFisher), streptomycin  
174 sulphate 100 $\mu$ g/ml (Sigma) and penicillin 75 $\mu$ g/ml (Sigma). To assess the effects of  
175 exogenous Strap protein on early follicle development, ovary fragments were treated  
176 with 0 (diluent only; control), 100 or 200ng/ml Human Unrip full-length recombinant  
177 protein (designated rhStrap; ab132509; Abcam, Cambridge, UK). For  
178 immunoneutralisation experiments, media was supplemented with either 1 or  
179 10 $\mu$ g/ml rabbit anti-Strap (AB1) IgG (AV48038; Sigma). Control wells were instead  
180 supplemented with 0, 1 or 10 $\mu$ g/ml of non-immune rabbit IgG (Vector). For the siRNA  
181 experiments, ovary fragments were initially maintained in culture media alone for  
182 three days before supplementation with either 1 $\mu$ M Accell mouse Strap siRNA (E-  
183 045977; Dharmacon, ThermoFisher, UK), or 1 $\mu$ M Accell non-targeting siRNA (D-  
184 001910-10; Dharmacon). Both siRNA contain a mixture of four oligonucleotides  
185 provided as a single reagent. Additional control wells without siRNA were also  
186 included. **One well from each group was collected at the termination of culture for  
187 analysis of Strap mRNA expression by qPCR according to the procedures described  
188 above.** All cultures were carried out three times (n=3 ovaries/group/experiment). After  
189 three days at 37°C and 5% CO<sub>2</sub>, ovary fragments would adhere and spread on the  
190 base of the well. This permits visualisation of oocytes with a standard inverted light  
191 microscope (Olympus CKX41 with a Nikon camera DS-Fi1). Using this approach, we  
192 were able to capture images and measure oocyte diameters using ImageJ  
193 (<http://imagej.nih.gov/ij/>) to monitor growth at specified time points during culture.

194 Only oocytes with a clearly identifiable boundary were counted and measured. At the  
195 end of culture, some ovary fragments were briefly fixed in 10% NBF (Sigma), washed  
196 in PBS, treated with 0.25% (v/v) triton X-100 (Sigma) for 15 minutes, and blocked in  
197 CAS-Block (ThermoFisher) before applying a solution of rabbit anti-Ddx4 (5µg/ml;  
198 ab13840; Abcam) and mouse anti-Amh (1:400; MCA2246; AbD Serotec, Oxford, UK)  
199 followed by overnight incubation at 4°C. Tissues were washed in PBS before  
200 applying secondary antibodies as above. After further washing in PBS, tissues were  
201 counterstained in 10µg/ml DAPI (Sigma) for 10 minutes before being replaced by  
202 PBS. Images were recorded using an Olympus IX73 inverted microscope.

203

#### 204 *Classification of oocytes*

205 Oocytes from ovary fragment cultures were classified as non-growing, transitional or  
206 growing using morphological parameters obtained from haematoxylin and eosin  
207 stained sections. One section was analysed from each ovary from the following ages:  
208 d4 (n=5), d8 (n=8), d13 (n=5), d16 (n=7). Digital images of each section were  
209 recorded with a light microscope and were imported to ImageJ. Oocyte diameters  
210 were measured in all follicles that had a clearly identifiable oocyte nucleus. Follicles  
211 were classified as non-growing (primordial) if it had a single layer of relatively flat  
212 granulosa cells. Transitional follicles had of a mixture of flat and enlarged/cuboidal  
213 granulosa cells in a single layer. Follicles were considered to be growing if they  
214 contained at least one complete layer of enlarged granulosa cells and a visually  
215 larger oocyte. After plotting these distributions (Suppl. Fig 1), we considered that any  
216 oocyte smaller than one standard deviation from the mean of the transitional  
217 category to be defined as non-growing (<18.3µm), and conversely any oocyte larger  
218 than one standard deviation from the mean of the transitional category to be defined  
219 as growing (>25.7µm). Any oocyte diameter between these boundaries was defined  
220 as transitional (18.3µm-25.7µm). Across all ages, a total of 992 non-growing, 292  
221 transitional and 331 growing preantral follicles were measured.

222

223 *Preantral follicle culture*

224 Preantral follicles from d16 ovaries (n=7) were mechanically dissected in drops of  
225 isolation media using acupuncture needles as previously described (Fenwick *et al.*  
226 2013). Follicles of similar size and appearance were transferred into 96-well plates  
227 with each well containing 100µl MEM-α (ThermoFisher) supplemented with 0.1%  
228 (w/v) BSA (Sigma), 75µg/ml penicillin (Sigma), 100µg/ml streptomycin sulphate  
229 (Sigma) and insulin-transferrin-sodium selenite (ITS; Sigma; 5µg/ml, 5µg/ml, 5ng/ml,  
230 respectively). A single follicle was placed into each well; 8 wells contained media with  
231 200ng/ml rhStrap (Abcam), while 8 contained media with diluent only (control). Each  
232 plate contained follicles from a single ovary, which were maintained for 72 hours at  
233 37°C and 5% CO<sub>2</sub>. Follicles were imaged daily using an Olympus CKX41 inverted  
234 microscope and diameters were determined from perpendicular measurements taken  
235 from the basement membrane of the follicle using ImageJ. Any follicles containing an  
236 oocyte that showed signs of degeneration or extrusion were excluded from the  
237 analysis.

238

239 *Statistical analyses*

240 For qPCR data, comparisons between d4, d8 and d16 samples (n=5 or 6 ovaries per  
241 age group as stated) were analysed by a Kruskal-Wallis test followed by a post-hoc  
242 Dunn's multiple comparisons test. For cultured ovarian fragments, proportions were  
243 grouped by treatment and stage and analysed using a two-way ANOVA (n=3 cultures  
244 per treatment). Post-hoc Bonferroni multiple comparisons tests were used to identify  
245 specific differences (effect of treatment) within each stage (non-growing, transitional,  
246 growing). For each experiment, growing oocytes from all three cultures were  
247 considered together (number as stated in figure legends) and analysed using a  
248 Kruskal-Wallis and Dunn's multiple comparisons test to determine effect of treatment  
249 at each time point. Preantral follicle growth was analysed by a two-way repeated

250 measures ANOVA (n=7 ovaries), with a Bonferroni's multiple comparisons test to  
251 determine effects at each time point. All analyses were performed using Prism  
252 (v6.0d; Graphpad) with differences considered significant if  $P < 0.05$ .  
253

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254 **Results:**

255

256 *Expression of Strap and Smads during early follicle development*

257

258 Ovaries from 4, 8 and 16 day old mice (d4, d8, d16) enriched with increasing  
259 proportions of developing preantral follicles were used to initially determine relative  
260 levels of mRNA of the BMP signalling Smads - *Smad1*, *Smad5* and *Smad9* (also  
261 known as *Smad8*), the TGFB signalling Smads – *Smad2* and *Smad3*, the co-Smad –  
262 *Smad4*, the inhibitory Smads - *Smad6* and *Smad7*, and *Strap* (Fig 1). Transcript  
263 levels of *Smad1* and *Smad5* were significantly higher in d16 ovaries relative to d4  
264 ( $P<0.01$  and  $P<0.001$ , respectively), while *Smad9*, *Smad2* and *Smad4* did not vary  
265 between age groups. Conversely, *Smad3*, *Smad7* and *Strap* were significantly lower  
266 in d16 ovaries relative to d4 ( $P<0.05$  each). The level of *Smad6* mRNA was  
267 increased in d8 ovaries relative to d4 only ( $P<0.05$ ).

268

269 To further evaluate the relationship between Strap and the TGFB receptor regulated  
270 Smads during early follicle development; proteins were immunolocalised in sections  
271 of d4, d8 and d16 ovaries (Fig 2). In d4 ovaries densely populated with primordial  
272 follicles, *Smad2/3* staining was evident in the flattened granulosa cells of single  
273 layered follicles, being more intense in cells of primordial and transitional than early  
274 growing (primary staged) follicles. Strap was also detectable in granulosa cells of  
275 small, single layered follicles and was strong in primordial oocytes. In d8 ovaries,  
276 *Smad2/3* and Strap were both detectable in granulosa cells of primary follicles and  
277 those that had begun to develop a second layer. In d16 ovaries, *Smad2/3* and Strap  
278 remained co-localised in granulosa cells of multi-layered preantral follicles, although  
279 the intensity of *Smad2/3* staining was weaker in comparison to smaller follicles. Strap  
280 was also detectable in extra-follicular stromal cells, although some of this could be  
281 accounted for by non-specific binding of rabbit IgG (Fig 2).

282

283 *Effect of Strap supplementation on early follicle development*

284

285 To reconcile the expression of intracellular TGFB pathway components in small  
286 follicles, we used a culture model to assess the effect of exogenous Strap on early  
287 growth. Ovaries from d4 mice were initially dissected and into fragments and  
288 maintained *in vitro* for 3 days to allow them to adhere and spread across the base of  
289 the well. This permits visualisation of the oocytes by light microscopy, which can be  
290 used as an indicator of follicle growth. After supplementation with 0, 100 or 200ng/ml  
291 recombinant human (rh) Strap protein, oocyte diameters were measured 4 and 7  
292 days later (Fig 3). At day 4, the proportion of oocytes classified as non-growing was  
293 similar across the three groups. The proportion of oocytes classified as transitional  
294 was slightly reduced in ovary fragments exposed to 200ng/ml rhStrap vs control  
295 ( $P<0.01$ ). Conversely, the proportion of oocytes classified as growing was slightly  
296 elevated in ovary fragments exposed to 200ng/ml rhStrap vs control ( $P<0.001$ ) (Fig  
297 3a). Similar trends were evident after 7 days of treatment: the proportion of non-  
298 growing oocytes was equivalent, whereas a reduction in the proportion of transitional  
299 oocytes was evident for ovary fragments exposed to 200ng/ml rhStrap relative to  
300 both 0 and 100ng/ml groups ( $P<0.05$  and  $0.01$ , respectively). The proportion of  
301 oocytes classified as growing was higher in ovary fragments exposed to 200ng/ml vs  
302 100ng/ml rhStrap ( $P<0.05$ ) (Fig 3b).

303

304 When only growing oocytes were considered (i.e.  $>25.7\mu\text{m}$ ), the median diameter  
305 was greater in wells exposed to 200ng/ml rhStrap at both 4 and 7 days ( $P<0.01$  vs  
306 0ng/ml). The median diameter of growing oocytes was also increased in fragments  
307 exposed to 100ng/ml vs 0ng/ml ( $P<0.05$ ) at 7 days (Fig 3c).

308

309 To determine whether an increase in oocyte diameter could be associated with  
310 markers of follicle development, some wells were stained with antibodies to clearly  
311 delineate oocytes (Ddx4) and to identify granulosa cells from growing preantral  
312 follicles (Amh) (Fig 3d). Large Ddx4-positive oocytes were mostly surrounded by  
313 Amh positive cells, indicating that the culture system was able to support primordial  
314 follicle activation and early preantral development. Although not quantified, Amh  
315 staining appeared to be more prevalent in the 200ng/ml rhStrap treated in  
316 comparison to controls.

317

#### 318 *Effect of Strap inhibition on early follicle development*

319 The same ovary fragment model was then used to assess the effects of Strap protein  
320 neutralisation on oocyte growth (Fig 4). Fragments were exposed to rabbit anti-Strap  
321 IgG (A-S) or non-immune rabbit IgG (N-I) or no IgG (Control) for 7 days. For those  
322 treated with 1µg/ml A-S, there was no effect on the proportion of oocytes classified  
323 as non-growing, transitional or growing after 4 days (Fig 4a). However, after 7 days,  
324 there was a significant reduction in the proportion of non-growing oocytes with a  
325 corresponding increase in the proportion of transitional and growing oocytes when  
326 compared with N-I or Control ( $P<0.001$  each) (Fig 4b). When ovary fragments were  
327 treated with 10µg/ml A-S, a reduction in the proportion of non-growing follicles was  
328 evident at 4 days, with a corresponding increase in the proportion of growing oocytes  
329 ( $P<0.001$  vs N-I or Control) (Fig 4c). The same trend was also found at 7 days (Fig  
330 4d).

331

332 When only growing oocytes were considered, there was no detectable difference in  
333 the median diameter of oocytes treated with 1µg/ml A-S after 4 days; however, after  
334 7 days the median diameter was increased relative to Control ( $P<0.05$ ) and N-I  
335 ( $P<0.01$ ) (Fig 4e). By comparison, the median diameter of growing oocytes was  
336 increased at 4 days when exposed to a higher concentration (10µg/ml) of A-S

337 relative to Control ( $P<0.05$ ) or N-I ( $P<0.05$ ). This increase was also evident at 7 days  
338 ( $P<0.01$  vs Control or  $P<0.001$  vs N-I) (Fig 4f).

339

340 In addition to protein inhibition, we then evaluated the effect of *Strap* mRNA inhibition  
341 using small interfering RNA (siRNA) in the same culture model (Fig 5). Ovarian  
342 fragments were exposed to  $1\mu\text{M}$  oligonucleotides targeting *Strap* mRNA, or  $1\mu\text{M}$   
343 non-targeting oligonucleotides (NT siRNA), or control ( $0\mu\text{M}$ ). After 4 days of  
344 treatment (7 days in culture), *Strap* mRNA was reduced by approximately 50% in the  
345 targeting vs the non-targeting groups and control groups; however, the difference  
346 was only significant between the targeting and non-targeting groups ( $P<0.05$ ; Suppl.  
347 Fig 2). Based on image analysis, there was a reduction in the proportion of non-  
348 growing oocytes in fragments exposed to *Strap* siRNA relative to both control and NT  
349 siRNA ( $P<0.001$ ). This corresponded with an increase in the proportion of growing  
350 oocytes in those fragments exposed to *Strap* siRNA relative to controls and NT  
351 siRNA ( $P<0.001$ ) (Fig 5a). The diameter of growing oocytes was also significantly  
352 increased relative to control ( $P<0.05$ ) and NT siRNA groups ( $P<0.01$ ) (Fig 5b). The  
353 increase in oocyte size in fragments exposed to *Strap* siRNA was also associated  
354 with many *Amh* positive granulosa cells (Fig 5c).

355

#### 356 *Effect of Strap supplementation on preantral follicle growth*

357 We then looked at the effect of exogenous *Strap* on isolated growing follicles.  
358 Preantral follicles from d16 mice were cultured either in the absence (Control) or  
359 presence of  $200\text{ng/ml}$  rh*Strap* for 72 hours (Fig 6). Follicles exposed to rh*Strap* grew  
360 at a faster rate than controls; meaning treated follicles were significantly larger in  
361 diameter at 48 hours ( $P<0.01$ ) and 72 hours ( $P<0.001$ ) relative to untreated follicles  
362 (Fig 6a). The morphological appearance of these cultured follicles was similar  
363 regardless of treatment group (Fig 6b).

364

365 **Discussion**

366

367 In this study we show that Strap, a TGFB pathway-regulating protein, is expressed in  
368 small follicles in the mouse ovary and is capable of influencing early follicle  
369 development. To examine the role of Strap on follicle growth we used an *in vitro*  
370 system where pieces of neonatal (d4) mouse ovaries enriched with small, non-  
371 growing follicles were monitored in conditions that aimed to reduce or increase Strap  
372 activity. In this context, inhibition of the protein by antibody neutralisation, or the  
373 transcript by RNA interference, caused a reduction in the proportion of non-growing  
374 follicles and a corresponding increase in the proportion of growing follicles. This  
375 shows the primary effect of Strap inhibition is to promote the activation of non-  
376 growing follicles. By comparison, when d4 ovaries were treated with exogenous  
377 Strap protein, there was no effect on the proportion of non-growing follicles.  
378 Interestingly, Strap protein seemed to promote the growth of follicles that had already  
379 begun to grow.

380

381 In other cell types, Strap is known to act by stabilising a complex between TGFBR1  
382 and Smad7 to effectively inhibit downstream R-Smad signalling (Datta & Moses  
383 2000) (Fig 7A). We therefore looked at R-Smad expression in relation to early follicle  
384 development. Initial results in this study confirmed previous observations that  
385 Smad2/3 are found in GCs of small, single-layered follicles (Xu *et al.* 2002, Fenwick  
386 *et al.* 2013). Smad2/3 protein expression is weak in growing follicles and similarly,  
387 the level of *Smad3* mRNA is relatively reduced in d16 ovaries containing many more  
388 growing follicles than younger d4 ovaries. We also previously showed that  
389 Smad1/5/8 protein is undetectable in small non-growing follicles and is very clearly  
390 expressed in GCs of growing preantral follicles (Fenwick *et al.* 2013). This is  
391 consistent with levels of *Smad1* and *Smad5* mRNA, which are increased in d16  
392 ovaries relative to d4. Together, the expression patterns of these R-Smads suggest a

393 stage-specific role for a sub-group of TGF $\beta$  ligands and receptors, with those that  
394 activate the Smad2/3 pathway as potentially key during the earliest stages. Since  
395 TGF $\beta$ s often promote the expression of factors that negatively regulate Smad  
396 signalling (Nakao *et al.* 1997, Stopa *et al.* 2000, Shi & Massague 2003, Gao *et al.*  
397 2013), we also looked at *Smad7* and *Strap* in immature mouse ovaries. In this model,  
398 *Smad7* and *Strap* transcript levels both followed a similar trend to *Smad3*, but not  
399 *Smad1*, *Smad5* or *Smad9* in d4-16 ovaries. In other cell types, Smad7 can inhibit the  
400 Type 1 receptors upstream of both Smad2/3 and Smad1/5/9 (Hanyu *et al.* 2001,  
401 Kamiya *et al.* 2010). The suggestion from the expression data is that Strap and  
402 Smad7 may be associated with the Smad2/3 pathway rather than Smad1/5/9.

403

404 Using immunohistochemistry the Strap protein appeared to localise to GCs of single  
405 and multi-layered preantral follicles consistent with a role for Strap in modulating  
406 Smad2/3 signalling. We did not see a reduction in Strap protein in growing follicles in  
407 the same way that Smad2/3 was reduced, although others have reported that the  
408 Strap protein is highly stable (Reiner & Datta 2011). The functional role of Strap in  
409 follicle development and fertility has not been determined since knocking out the  
410 gene in mice causes embryonic lethality (Chen *et al.* 2004). Experimental reduction  
411 of Strap in our culture model resulted in an increased proportion of follicles initiating  
412 growth. Thus, it is possible that a loss of Strap permits increased Smad2/3 signalling  
413 in GCs of small follicles. The role of Smad2/3 signalling in small single-layered  
414 follicles is not clear, although mice lacking exon 8 of *Smad3* have significantly more  
415 non-growing follicles and fewer growing follicles than wild-type mice at 3 months of  
416 age (Tomic *et al.* 2004). In human cultured human ovarian cortical tissues, treatment  
417 of with a low concentration of activin inhibited follicle activation, while a higher  
418 concentration increased activation (Ding *et al.* 2010). Increased Smad2/3 signalling  
419 may therefore be an important prerequisite for follicles to initiate growth.

420

421 In the same culture model we observed a positive influence of recombinant Strap  
422 protein on growing follicles. Interestingly, there was no effect of exogenous Strap on  
423 the small, non-growing follicles. The mechanism is not clear; however, the observed  
424 reduction in Smad2/3 expression in follicles with multiple layers of granulosa cells  
425 provides some suggestion that loss of this signalling pathway may be important for  
426 growing follicles. A recent study in mice showed that inhibition of TGFBR1 in a similar  
427 ovary culture model resulted in accelerated oocyte growth and GC proliferation  
428 (Wang *et al.* 2014). Therefore, the differential actions of Strap in small follicles may  
429 reflect the change in Smad2/3 expression observed between non-growing and  
430 growing follicles.

431

432 Strap also binds numerous proteins independent of the TGFB-Smad pathway (Fig  
433 7A). For example, Strap can interact with factors that promote proliferation (Seong *et al.*  
434 *et al.* 2005, Kashikar *et al.* 2011, Reiner *et al.* 2011), regulate the  
435 epithelial/mesenchymal phenotype (Kashikar *et al.* 2010) and inhibit apoptosis (Jung  
436 *et al.* 2010). For these reasons, elevated levels of Strap have been associated with  
437 tumourigenesis (Kim *et al.* 2007). Activation of the PI3 kinase/Akt pathway is known  
438 to promote early follicle development (Reddy *et al.* 2005, Liu *et al.* 2007, Reddy *et al.*  
439 2008, Adhikari & Liu 2009). Within this pathway, Strap is capable of binding PDK1,  
440 where it enhances phosphorylation of target substrates (Seong *et al.* 2005), including  
441 mTORC1, the activation of which is positively associated with early follicle  
442 development (Adhikari *et al.* 2010, Zhang *et al.* 2014). The observed increase in the  
443 proportion and size of growing follicles, as well as the increased rate of growth of  
444 isolated preantral follicles treated with recombinant Strap protein may reflect these  
445 interactions with Smad-independent pathways. Considering the stage-dependent  
446 effects that we observed by either increasing or decreasing Strap activity, we  
447 propose a model that explains how this factor may influence early follicle  
448 development, in accordance with the expression of TGFB-Smads (Fig 7B).

449

450 In this study we used a novel model of ovary culture to study the effects of Strap *in*  
451 *vitro*. Cutting the ovary into fragments causes loss of the original architecture but still  
452 allows follicles to grow within a heterogeneous environment of other follicles and  
453 ovarian cells. A similar model has been used to evaluate the effects of  
454 chemotherapeutics on small follicles (Maiani *et al.* 2012). Culturing whole rodent  
455 ovaries has been carried out for some time (Eppig & O'Brien 1996, O'Brien *et al.*  
456 2003); however, the ability of this system to support follicles developing multiple  
457 layers of GCs is sometimes limited. In addition, the density of whole ovaries prohibits  
458 clear visualisation of individual follicles *in vitro*. In our cultures, we were able to  
459 observe oocytes during culture and also show that growing oocytes were associated  
460 with GC-specific Amh expression, an additional marker of follicle growth. **Intriguingly,**  
461 **this model was amenable to various treatments including siRNA, as well as other**  
462 **macromolecules introduced to the culture media, such as recombinant protein and**  
463 **antibodies. The mechanism that allows the entry of these substances into the cells to**  
464 **interact with target proteins is not clear, but may involve non-specific receptor-**  
465 **independent endocytotic processes, such as clathrin-independent endocytosis or**  
466 **macropinocytosis (Maldonado-Baez *et al.* 2013, Mayor *et al.* 2014). Regardless of**  
467 **the mechanism involved, we observed clear dose-dependent effects of these**  
468 **macromolecules on oocyte growth in this system. Since these outcomes were**  
469 **evaluated in relation to controls indicates that the observed differences were specific**  
470 **to the macromolecule introduced to the system, and may therefore be an invaluable**  
471 **model for testing the effects other exogenous compounds on early follicle**  
472 **development *in vitro*.**

473

474 In summary we have shown that Strap is expressed and is capable of influencing  
475 small follicle growth in the mouse ovary *in vitro*. The mechanism of action is not clear  
476 but the co-expression of Smads during the early stages imply interactions with the

477 TGF $\beta$  pathway might be important, while the effect of Strap on growing follicles could  
478 be via alternative signalling pathways. The subtle influences of proteins that interact  
479 with these pathways potentially lead to more substantial effects on the rate and  
480 number of follicles that develop to more advanced stages.

481

482

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488

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- 649  
650  
651

652 **Table 1.** Primer sequences used for PCR assays in mouse ovaries.

Gene Symbol	Primer Sequence (5'→3')	GenBank Accession	Product Size (bp)
<i>Smad1</i>	Fwd: ACCTGCTTACCTGCCTCCT Rev: GCCTGAACATCTCCTCTGCT	NM_008539.3	114
<i>Smad2</i>	Fwd: CGTCCATCTTGCCATTAC Rev: GTCCATTCTGCTCTCCACCA	NM_001252481.1	102
<i>Smad3</i>	Fwd: GTCAAAGAACACCGATTCCA Rev: TCAAGCCACCAGAACAGAAG	NM_016769.4	154
<i>Smad4</i>	Fwd: CGGCGATTGTGCATTCTCAG Rev: CCTGGAAATGGTTAGGGCGT	NM_008540.2	209
<i>Smad5</i>	Fwd: CCTTGCTCATCTCCCTGTCT Rev: CCGTGAATCTCCTTTCTGTG	NM_001164041.1	173
<i>Smad6</i>	Fwd: TGCAACCCCTACCACTTCA Rev: GCTGGCATCTGAGAATTCA	NM_008542.3	180
<i>Smad7</i>	Fwd: AGTCAAGAGGCTGTGTTGCTGT Rev: CATTGGGTATCTGGAGTAAGGA	NM_001042660.1	130
<i>Smad9</i>	Fwd: GTCTGACCTTGCAGATGGCT Rev: TAGGTGCCAGGCTGAGAGAT	NM_019483.5	235
<i>Strap</i>	Fwd: GGCTACTTTCTGATCAGCGC Rev: CTGAGACCGCATCCCATACT	NM_011499.3	187

653

654

1 **Figure 1:**

2 Relative mRNA expression of *Smads* and *Strap* in juvenile mouse ovaries.  
3 Transcripts were amplified from cDNA derived from whole mouse ovaries by qPCR  
4 and normalised with endogenous *Atp5b* as described in the *materials and methods*.  
5 Fold changes (mean  $\pm$ SEM) in d8 and d16 ovaries are shown relative to d4. *Smad1-*  
6 *3, 5, 7*, n=6 ovaries each age; *Smad4, Smad6, Smad9 and Strap*, n=5 ovaries each  
7 age; \*P<0.05 vs d4, \*\*P<0.01 vs d4, \*\*\*P<0.001 vs d4, Dunn's multiple comparisons  
8 test.

9  
10 **Figure 2:**

11 Co-localisation of Smad2/3 and Strap in juvenile mouse ovaries highlighting  
12 relationship with these proteins and early follicle development. Smad2/3 is labelled in  
13 green (a,d,g) and Strap is labelled in red (b,e,h). Merged images (c,f,i) represent  
14 ovaries from mice at d4 (a-c), d8 (d-f) and d16 (g-i). Arrows, arrowheads and  
15 asterisks indicate primordial, primary and multi-layered preantral follicles,  
16 respectively. Control sections from d16 mice were incubated with mouse IgG (j),  
17 rabbit IgG (k) or dilution buffer (l) in place of the primary antibodies. All sections were  
18 counterstained with DAPI (blue). Scale = 100 $\mu$ m.

19  
20 **Figure 3:**

21 Effect of Strap supplementation on small follicle growth *in vitro*. Ovaries from d4 mice  
22 were cut into small fragments and maintained under standard culture conditions  
23 (Control) or supplemented with 100ng/ml (100) or 200ng/ml (200) recombinant  
24 human Strap (rhStrap). Oocytes were classified as non-growing, transitional or  
25 growing and proportions in each category are shown at day 3 (a) and day 6 of culture  
26 (b). Mean  $\pm$ 95% CI; n=3 cultures; \*P<0.05, \*\*P<0.01, Bonferroni's multiple  
27 comparisons test. At day 3 and 6 of culture, the median diameters (horizontal bars) of  
28 growing oocytes (>25.7 $\mu$ m) in each treatment group are plotted (c) \*P<0.05,

1 \*\*P<0.01, Dunn's multiple comparisons test (Day 3 n=101, 117, 164 and Day 6  
2 n=169, 221, 263 oocytes for C, 100 and 200 groups, respectively). At day 6 some  
3 wells were co-stained with Ddx4 (green) to label oocytes and Amh (red) to label  
4 granulosa cells of growing follicles (d). Cell nuclei were counterstained with DAPI  
5 (blue). Scale bar = 50µm.

6

7 **Figure 4:**

8 Effect of Strap immuno-neutralisation on small follicle growth *in vitro*. Ovaries from d4  
9 mice were cut into small fragments and maintained under standard culture conditions  
10 (Control) or supplemented with non-immune IgG (N-I) or anti-Strap IgG (A-S).  
11 Oocytes were classified as non-growing, transitional or growing and proportions in  
12 each category are shown at day 4 (a) and day 7 (b) of culture with 1µg/ml  
13 supplementation and day 4 (c) and day 7 (d) of culture with 10µg/ml  
14 supplementation. Mean ±95% CI; n=3 cultures; \*\*\*P<0.001 vs Control and N-I  
15 groups, Bonferroni's multiple comparisons test. At day 4 and 7 of culture, the median  
16 diameters (horizontal bars) of growing oocytes (>25.7µm) in the presence of 1µg/ml  
17 (Day 4 n=65, 73, 89 and Day 7 n=81, 86, 101 oocytes for C, 1µg N-I and 1µg A-S  
18 groups, respectively) (e) and 10µg/ml (f) IgG are plotted (Day 4 n=96, 97, 117 and  
19 Day 7 n=102, 98, 123 oocytes for C, 10µg N-I and 10µg A-S groups, respectively).  
20 \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, Dunn's multiple comparisons test.

21

22 **Figure 5:**

23 Effect of Strap mRNA knock down on small follicle growth *in vitro*. Ovaries from d4  
24 mice were cut into small fragments and maintained under standard culture conditions  
25 (Control) or supplemented with non-targeting siRNA (NT siRNA) or siRNA  
26 complementary to Strap mRNA (Strap siRNA). Oocytes were classified as non-  
27 growing, transitional or growing and proportions in each category are shown for each  
28 treatment after 4 days (7 days of culture) (a). Mean ±95% CI; n=3 cultures;

1 \*\*\*P<0.001 vs Control and NT siRNA groups, Bonferroni's multiple comparisons test.  
2 At day 7 of culture, the median diameters (horizontal bars) of growing oocytes  
3 (>25.7µm) for the different groups are plotted (b). \*P<0.05, \*\*P<0.01, Dunn's multiple  
4 comparisons test (n=89, 80, 93 oocytes for C, NT siRNA and Strap siRNA groups,  
5 respectively). At day 7 some wells were co-stained with Ddx4 (green) to label  
6 oocytes and Amh (red) to label granulosa cells of growing follicles (c). Scale =  
7 100µm.

8

9 **Figure 6:**

10 Effect of Strap supplementation on preantral follicle growth *in vitro*. Preantral follicles  
11 were isolated from d16 mice and maintained under standard culture conditions  
12 (Control) or supplemented with 200ng/ml recombinant human Strap (rhStrap). 6-8  
13 follicles were included per group for each ovary and diameters measured daily. Only  
14 morphologically healthy follicles were included in the analysis. Growth is plotted as  
15 the difference in diameter relative to 0h and represents the mean (±95% CI) of 7  
16 ovaries (n=7) at each time point (a). \*\*P<0.01, \*\*\*P<0.001 vs control at the indicated  
17 time point. Bonferroni's multiple comparisons test. Examples of cultured preantral  
18 follicles are shown (b). Scale = 50µm.

19

20 **Figure 7:**

21 Regulation of the TGFB signalling pathway by Strap in the context of early follicle  
22 development. (A) Canonical TGFB signalling involves ligands such as Gdf9, TGFB1-  
23 3 or activins associating with Type I and II receptors, leading to recruitment and  
24 activation of R-Smads (Smad2/3) and Smad4 complexes that enter the nucleus to  
25 regulate gene transcription in association with other co-factors (triangle, pentagon).  
26 Strap inhibits the canonical pathway by forming a complex with the Type I receptor  
27 and Smad7. Strap can also interact with other pathways such as PI3K and MAPK to  
28 promote cell proliferation. (B) Granulosa cells of small single-layered follicles, which

1 are relatively slow growing, express Smad2/3. A reduction in Strap may facilitate  
2 canonical TGFB signalling and promote the transition of growth through the early  
3 follicle stages. Further follicle growth is associated with a reduction in Smad2/3,  
4 which may be facilitated by the ascribed role of Strap in inhibiting TGFB signalling  
5 and driving TGFB independent pathways associated with cell proliferation and follicle  
6 growth.

7

### 8 **Supplemental Figure 1**

9 Distributions of oocyte sizes in small follicles from sections of mouse ovaries.  
10 Follicles were classified and corresponding diameters were measured from H&E  
11 stained sections. One section was analysed from each ovary from the following ages:  
12 d4 (n=5), d8 (n=8), d13 (n=5), d16 (n=7). *Non-growing* oocytes were measured from  
13 primordial staged follicles consisting of a small oocyte surrounded by a single layer of  
14 relatively flat granulosa cells. *Transitional* oocytes were measured from follicles that  
15 also had a single layer of granulosa cells but some of which were no longer flat in  
16 appearance. These follicles were named as such because their growth status is  
17 ambiguous. *Growing* oocytes were measured from follicles that were clearly larger in  
18 appearance and had at least one complete layer of cuboidal granulosa cells - these  
19 included follicles classified as primary staged and larger. Individual points represent a  
20 single oocyte (n=992 non-growing, n=292 transitional, n=331 growing) (mean  $\pm$  s.d.).  
21 Considering the overlap in in oocyte size distributions between the three groups, our  
22 criteria for classifying oocytes in the culture experiments were based on the mean  $\pm$  1  
23 s.d. of the transitional group (i.e. above and below the shaded region indicated on the  
24 graph). Oocytes with a diameter of  $<18.3\mu\text{m}$  were assigned to the *non-growing*  
25 category,  $18.3\text{-}25.7\mu\text{m}$  were assigned as *transitional*, and oocyte diameters  $>25.7$   
26 were assigned as *growing*.

27

**1 Supplemental Figure 2**

2 Expression of *Strap* mRNA in samples of cultured neonatal mouse ovary fragments  
3 after treatment with siRNA. Ovaries from d4 mice were cut into small fragments and  
4 were cultured for 3 days (6-8 equivalent sized pieces per well). Cultures were then  
5 either maintained under standard conditions (Control) or supplemented with 1µM  
6 non-targeting siRNA (NT siRNA) or 1µM siRNA complementary to *Strap* mRNA  
7 (*Strap* siRNA). After 4 days of treatment, samples were pooled from 3 wells to obtain  
8 sufficient RNA from each group and processed for cDNA synthesis. Expression of  
9 *Strap* mRNA was determined in relation to the internal reference gene *Atp5b* and  
10 expressed as fold change relative to Control group using the formula  $2^{-\Delta\Delta CT}$  as  
11 described in *Materials and Methods*. Fold changes (mean ±SEM) are shown for 4  
12 technical replicates (n=4). \*P<0.05, One-way ANOVA and Bonferroni's multiple  
13 comparisons test.

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Figure 1

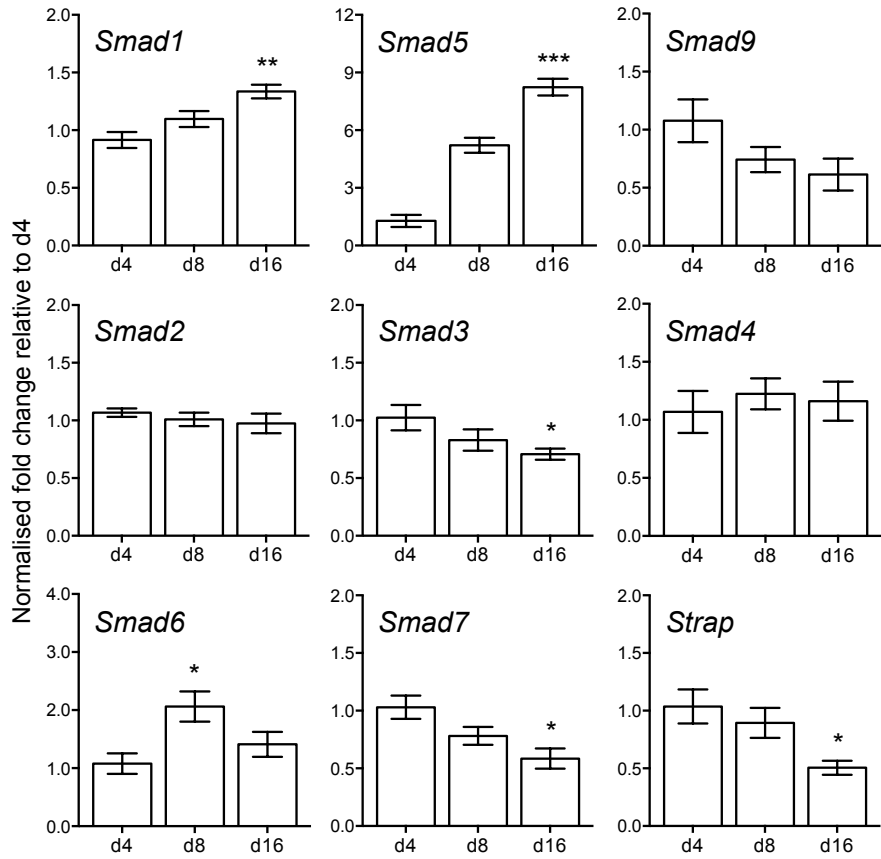


Figure 2

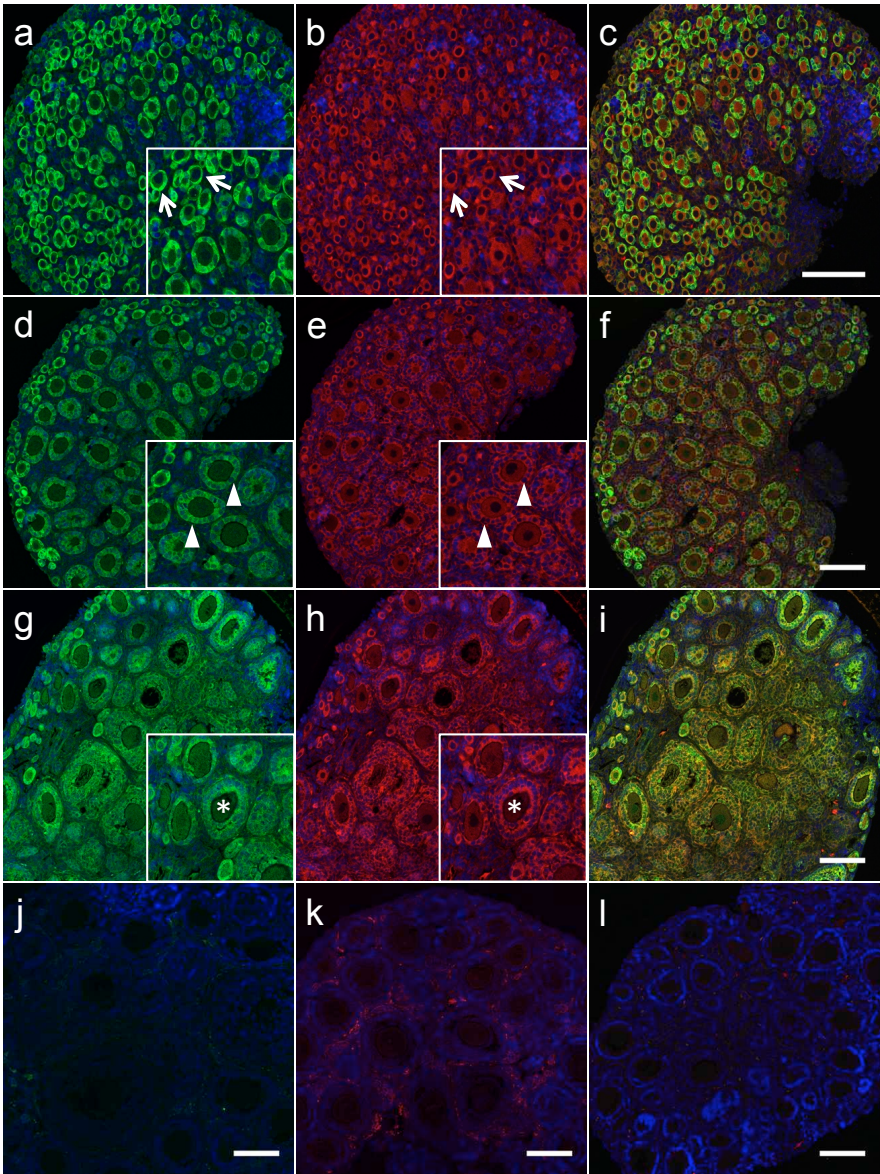


Figure 3

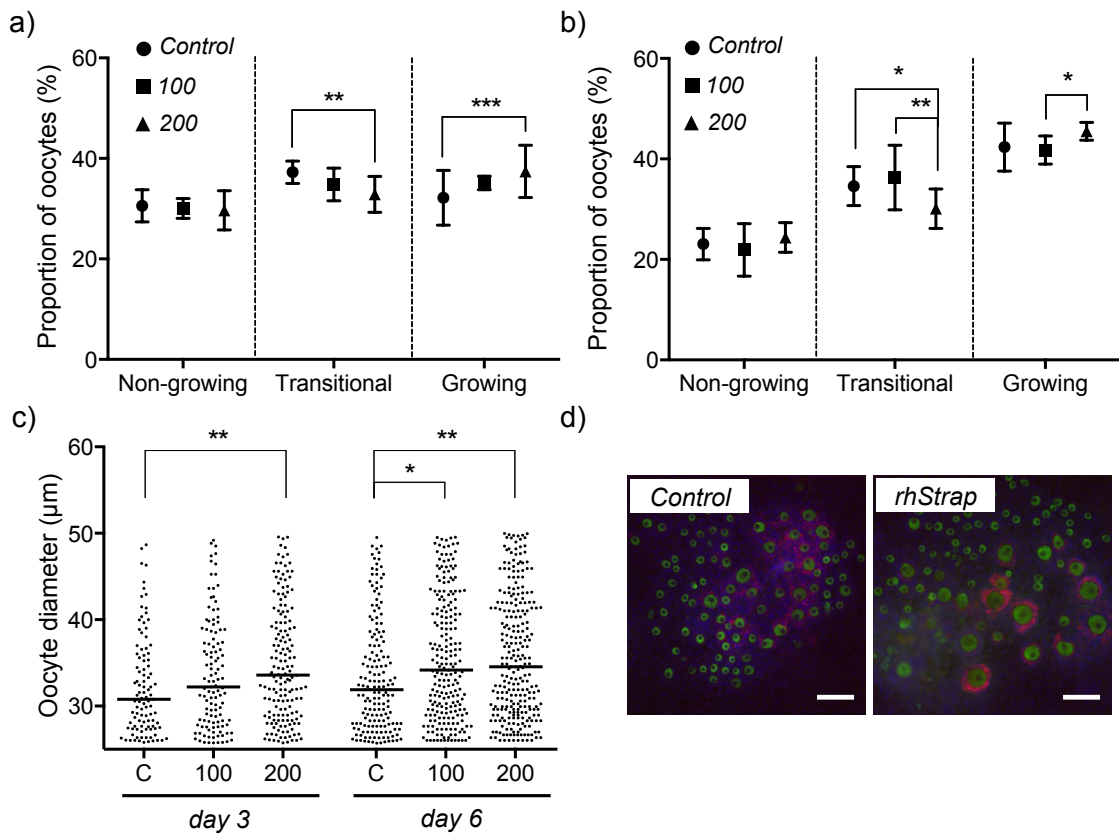


Figure 4

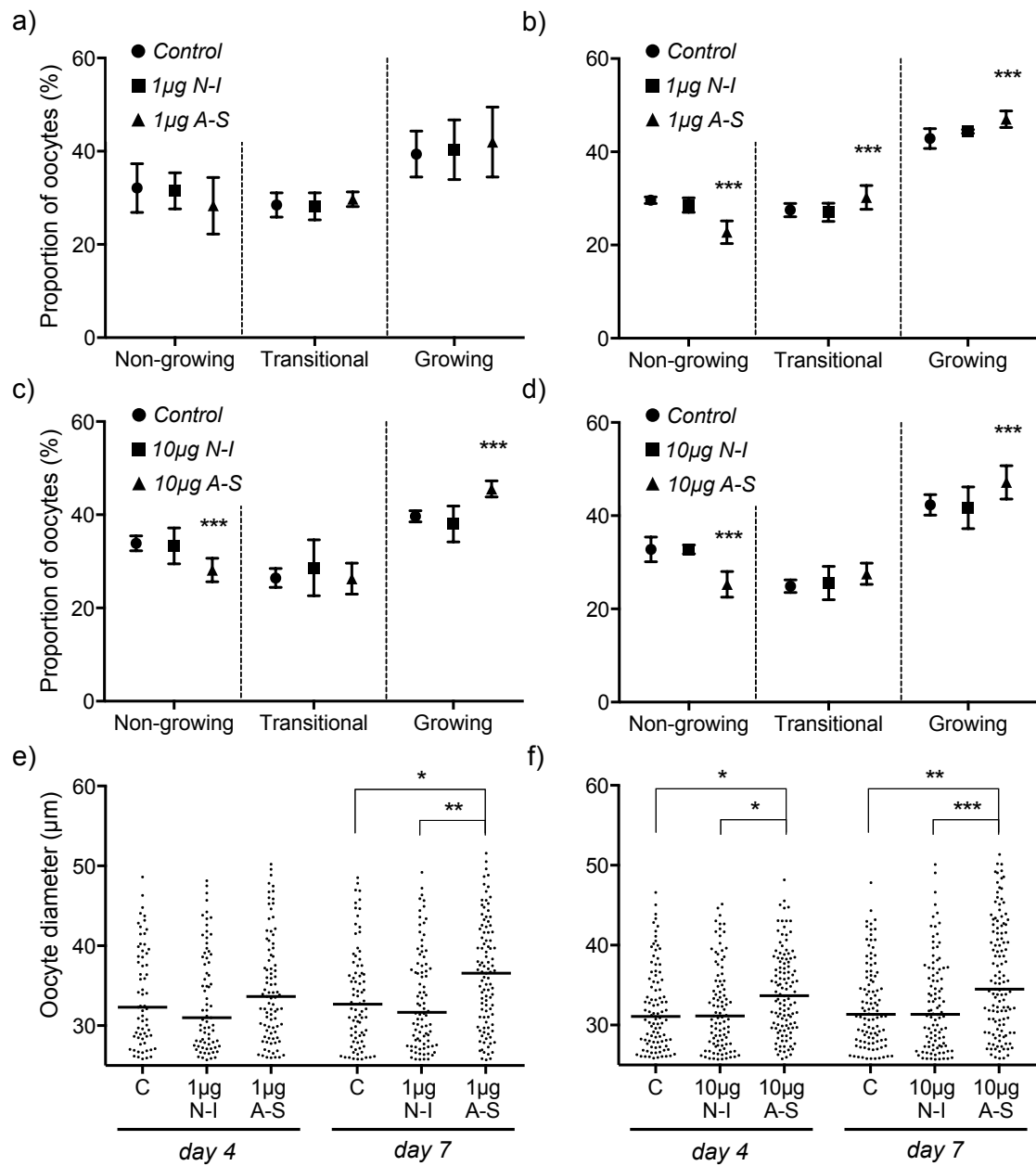


Figure 5

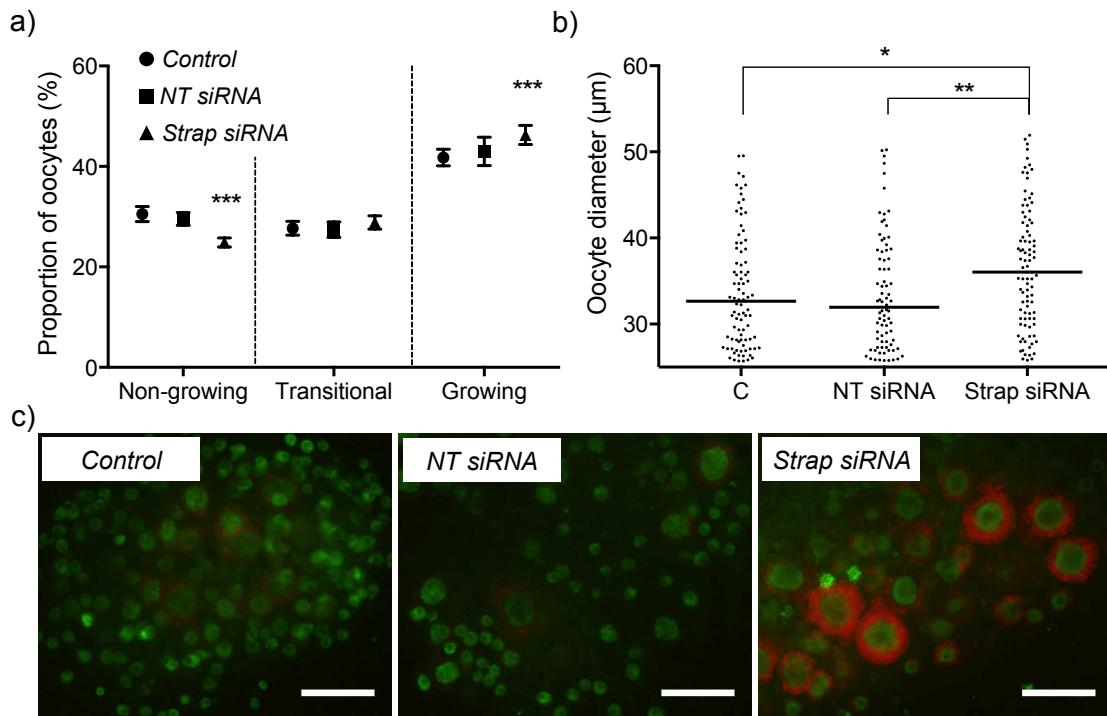
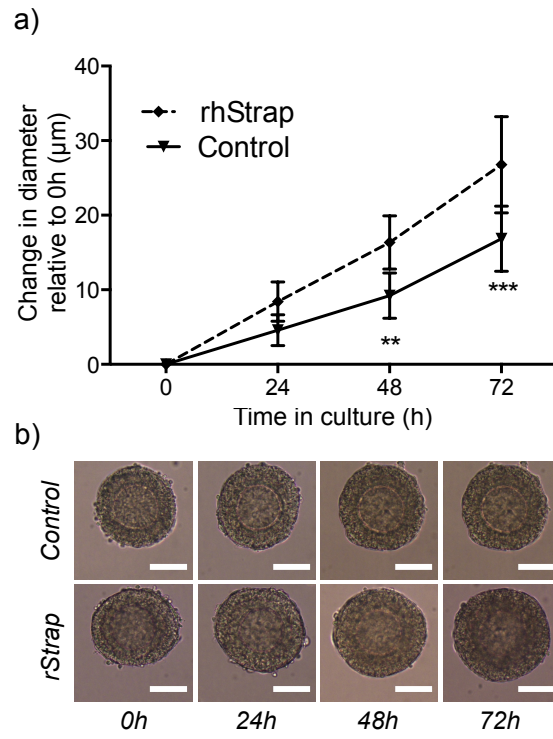
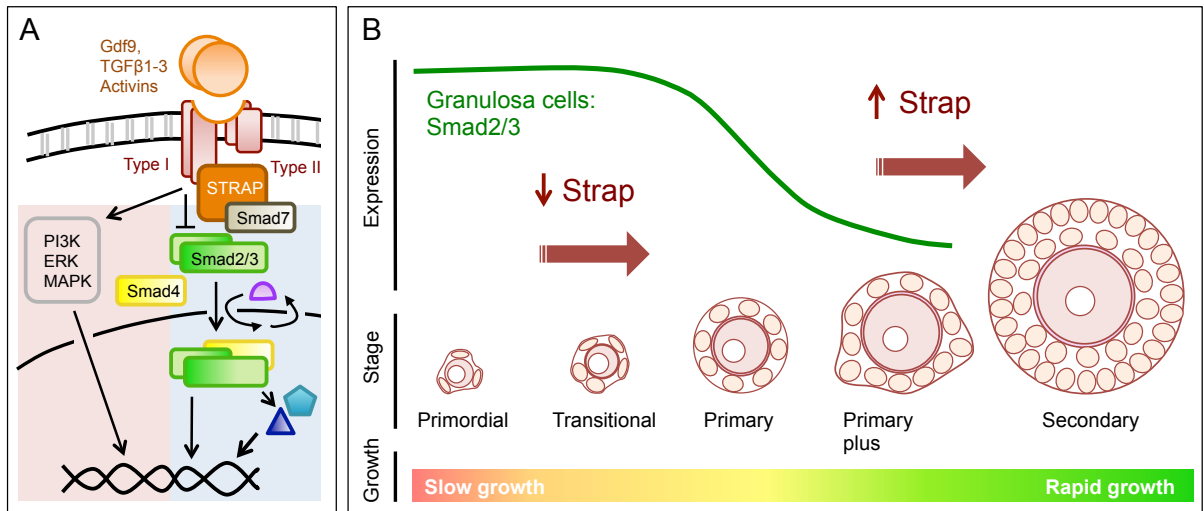


Figure 6

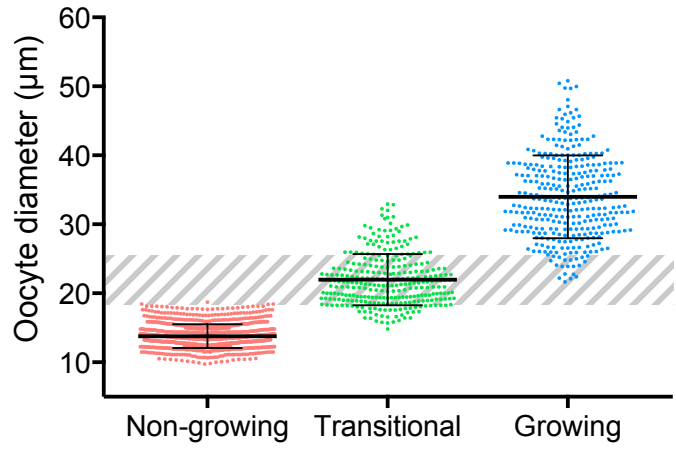


M

Figure 7

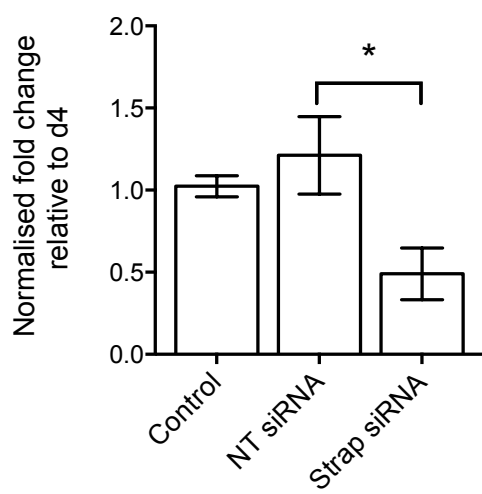


Supplementary Figure 1



For Review Only

Supplementary Figure 2



For Review Only

**Supplementary Table 1** Characteristics of qPCR assays

Gene	Primer efficiency (CT range)*	Intra-assay variation (%CV)**	Range of CT values across all ovary samples (d4, d8, d16)	
			Min	Max
<i>Smad1</i>	98% (26.5-36)	1.71	25.0	29.3
<i>Smad2</i>	109% (26.5-31)	2.70	25.6	29.7
<i>Smad3</i>	99% (27-36)	0.75	23.7	27.9
<i>Smad4</i>	105% (24-33)	4.88	25.7	28.8
<i>Smad5</i>	103% (27-34.5)	0.91	24.1	30.1
<i>Smad6</i>	89% (30-35)	0.84	28.3	33.0
<i>Smad7</i>	109% (27.5-34)	1.00	29.0	32.6
<i>Smad9</i>	103% (29-35.5)	1.82	30.1	33.0
<i>Strap</i>	95% (24.5-32)	0.68	25.6	27.9

\*Primer efficiency was evaluated using a dilution series of adult mouse ovary cDNA in triplicate. Mean efficiency (%) was determined from the slope of the log-linear portion of the curve (in parentheses) using the equation  $(10^{-1/\text{slope}} - 1) * 100$ .

\*\*Intra-assay variation was calculated from CT values from each of the dilution standards and is presented as mean %CV across the curve.