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A central role for cAMP/EPAC/RAP/PI3K/AKT/CREB signaling in LH-induced follicular Pgr expression at medaka ovulation

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Running title: A signaling cascade for medaka Pgr induction.

Summary sentence: EPAC/RAP/PI3K/AKT/CREB signaling mediates LH-induced cAMP signaling to induce medaka Pgr expression in ovulating follicles.

Keywords: teleost, medaka, LH, ovulation, PGR, EPAC/RAP/PI3K/AKT/CREB signaling

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32 **Abstract**

33

34 Nuclear progestin receptor (PGR) is a ligand-activated transcription factor that has been
35 identified as a pivotal mediator of many processes associated with ovarian and uterine
36 function, and aberrant control of PGR activity causes infertility and disease including
37 cancer. The essential role of PGR in vertebrate ovulation is well recognized, but the
38 mechanisms by which PGR is rapidly and transiently induced in preovulatory follicles
39 after the ovulatory LH surge are not known in lower vertebrates. To address this issue, we
40 utilized the small freshwater teleost medaka *Oryzias latipes*, which serves as a good
41 model system for studying vertebrate ovulation. In the *in vitro* ovulation system using
42 preovulatory follicles dissected from the fish ovaries, we found that inhibitors of EPAC
43 (brefeldin A), RAP (GGTI298), PI3K (Wortmannin), AKT (AKT inhibitor IV), and
44 CREB (KG-501) inhibited LH-induced follicle ovulation, while the PKA inhibitor H-89
45 had no effect on follicle ovulation. The inhibitors capable of inhibiting follicle ovulation
46 also inhibited follicular expression of Pgr and matrix metalloproteinase-15 (Mmp15), the
47 latter of which was previously shown to not only be a downstream effector of Pgr but also
48 a proteolytic enzyme indispensable for follicle rupture in medaka ovulation. Further
49 detailed analysis revealed for the first time that the cAMP/EPAC/RAP/PI3K/AKT/CREB
50 signaling pathway mediates the LH signal to induce Pgr expression in preovulatory
51 follicles. Our data also showed that phosphorylated Creb1 is a transcription factor
52 essential for *pgr* expression and that Creb1 phosphorylated by Akt1, rather than PKA,
53 may be preferably used to induce *pgr* expression.

54

55

56 **Introduction**

57

58 Nuclear progesterin receptor (PGR) is a progesterin-activated transcription factor that is
59 expressed primarily in female reproductive tissues, immune and neuronal systems [1-3].
60 PGR, which is luteinizing hormone (LH)-inducible, shows pleiotropic effects on
61 reproductive processes, and therefore, aberrant control of PGR action can not only cause
62 infertility but also results in various diseases associated with the organs, such as ovarian
63 and uterine cancer and endometriosis [1, 4]. Thus, an understanding of precise role of
64 PGR in normal function of the reproductive organs would be useful from a therapeutic
65 point of view.

66

67 Investigations of PGR have been intensively carried out in relation to ovulation [1, 5-11].
68 Since PGR is transiently and rapidly induced in the granulosa cells of preovulatory
69 follicles immediately after the LH surge and because it subsequently provokes the
70 expression of many ovulation-related genes in the cells, studies to date on PGR have
71 sought to either identify PGR-regulated genes/proteins or to unveil the mechanism and
72 signaling pathway by which LH induces PGR. In the former studies, many genes/proteins
73 have been documented to be expressed under the control of PGR expressed in the follicle
74 that is destined for ovulation [7, 8, 12-17]. On the other hand, attaining an understanding
75 of the signaling pathway and regulatory mechanism underlying PGR expression in LH-
76 stimulated preovulatory follicles still remains a challenge. Nevertheless, accumulating
77 evidence indicates that in the preovulatory follicle subjected to the ovulatory LH surge,
78 cAMP signaling evoked as a result of LH receptor activation sequentially flows via
79 protein kinase A (PKA), epidermal growth factor (EGF)-like factors/ EGF receptor,

80 ERK1/2 (also known as MAPK3/1) and inositol trisphosphate (IP3)/ diacylglycerol
81 (DAG). PGR is presumed to be expressed downstream of these mediators [15, 18-23]. In
82 addition, the requirement of nuclear receptor-interacting protein 1 (NRIP1) [24] and
83 activation of the $G\alpha_q/11$ pathway [25] has been reported for PGR expression. One report
84 investigated the expression of PGR in granulosa cells using an intact PGR promoter, with
85 a focus on the LH inducibility of PGR [26]. The study revealed that LH activates Sp1/Sp3
86 binding sites in the PGR proximal promoter, but the molecular mechanisms by which this
87 activation occurs leaves unanswered questions.

88

89 In all vertebrates, the ovary is principally regulated by the hypothalamus-pituitary-
90 gonadal axis [27, 28]; thus, the growth and proliferation of oogonia, their development to
91 the oocyte stage, and their eventual release from the ovary are believed to be under similar
92 endocrine control. These considerations, together with the general concept that there are
93 very few differences between teleosts and mammals at the molecular level [29], have
94 encouraged the use of teleost models to understand ovary physiology conserved across
95 vertebrates. Teleosts have been valuable animal models for examining the effects of
96 natural environmental changes [30-32]. It is also known that environmental contaminants
97 affect LH signaling, resulting in disruption of fertility in the wild teleost population. To
98 understand how such contaminants cause infertility in teleosts, elucidation of the LH
99 signaling pathway is a necessary task.

100

101 In teleosts, *Pgr* has also emerged as an important transcription factor for ovulation [6, 7,
102 33-35]. Notably, recent studies using *pgr* knockout zebrafish have revealed that fish
103 exhibit anovulation and infertility and that, as in mammals, the expression of many

104 ovulation-related genes is changed in KO fish compared with wild type fish [6]. Previous
105 studies using a freshwater teleost medaka, which serves as a good model for ovulation
106 study [36], have revealed that membrane-type 2 matrix metalloproteinase 2 (official name,
107 Mmp15) is indispensable for follicle rupture during medaka ovulation and that its
108 expression is drastically induced before ovulation in the pre- and/or peri-ovulatory
109 follicles of the fish ovary under the control of Pgr [34, 36, 37]. In addition, follicular *pgr*
110 expression was induced in an LH- and cAMP-dependent manner [33]. However, similar
111 to mammals, little is known about the intracellular signaling pathways leading from the
112 LH surge to *pgr*/Pgr expression in the ovarian follicles of the fish. With this in mind, we
113 address the currently ill-defined problem of the LH-elicited signaling pathways that
114 eventually lead to *pgr*/Pgr expression using a medaka model. Here, we report a novel
115 finding that an exchange protein directly activated by cAMP (Epac, official name,
116 Rapgef), which is a multidomain protein that functions as a cAMP-activated exchange
117 factor for the small G-proteins Rap1 and Rap2 [38-41], acts as a direct downstream
118 effector of cAMP increased in the granulosa cells of LH-stimulated preovulatory follicles.
119 Further analysis revealed that Epac1 activated with cAMP activates Rap1, which
120 subsequently activates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, followed
121 by phosphorylation of the transcription factor Creb1 and eventually leading to *pgr*
122 expression in the follicles. This signaling pathway that induces *pgr* expression in the
123 teleost ovary is completely different from that currently presumed for mammalian species.

124

125 **Materials and methods**

126

127 **Medaka culture and tissue preparation**

128

129 Adult medaka (*Oryzias latipes*), strain himedaka (orange-red variety), were purchased
130 from a local dealer. The fish were maintained and acclimated to the artificial reproductive
131 conditions, as previously described [36]. Under the conditions, females usually ovulate
132 every day just before the onset of light. In the present study, the start of the light period
133 was set as ovulation hour 0. Preovulatory follicles destined to ovulate (≥ 1.0 mm,
134 postvitellogenic phase, stage IX-X) were isolated as previously described [37]. Separation
135 of follicle layers from follicles was performed as previously described [36]. The
136 experimental procedures used in the present study were approved by the Committee of
137 the Experimental Plants and Animals, Hokkaido University.

138

139 ***In vitro* follicle culture and ovulation**

140

141 *In vitro* follicle culture was performed as previously described [42]. An outline of the
142 experiments is shown in Figure 1A. Preovulatory follicles, that had not yet been exposed
143 to an endogenous LH surge, were isolated from two to four fish ovaries 22 h before
144 ovulation (-22 h-follicles), pooled, and then divided into control and test groups. The
145 follicles (approximately 20–25 follicles per group) were incubated at 26 °C in 90% M199
146 medium (pH 7.4) in the presence of medaka recombinant LH (rLh) (100 µg/ml) with or
147 without specific inhibitors. The chemicals used were N-(cis-2-phenyl-cyclopentyl)
148 azacyclotridecan-2-imine-hydrochloride (MDL 12330A) (1-50 µM) (Calbiochem/Merck
149 KGaA, Darmstadt, Germany), NKY80 (adenylyl Cyclase Type V Inhibitor) (1-200 µM)
150 (Cayman, Ann Arbor, MI), H-89 (1-200 µM) (Santa-Cruz, Dallas, TX), PKA inhibitor 14-

151 22 amide (1-50 μ M) (Sigma-Aldrich, St. Louis, MO), brefeldin A (1-50 μ M) (Sigma-
152 Aldrich), EPAC 5376753 (1-100 μ M) (Focus Biomolecules, Plymouth Meeting, PA),
153 Wortmannin (1-50 μ M) (Calbiochem/Merck), GGTI 298 (1-200 μ M) (Sigma-Aldrich),
154 LY294002 (1-20 μ M) (Calbiochem/Merck), AKT inhibitor IV (1-50 μ M)
155 (Calbiochem/Merck), AKT inhibitor (1-200 μ M) (Santa-Cruz), naphthol AS-E phosphate
156 (KG-501) (1-20 μ M) (Sigma-Aldrich), CBP-CREB interaction inhibitor (1-500 μ M)
157 (Calbiochem/Merck), 8-bromoadenosine 3', 5'-cyclic monophosphate (8-Br-cAMP) (1
158 μ M) (Sigma-Aldrich), forskolin (1 μ M) (Sigma-Aldrich), U0126 (25 μ M) (Wako, Osaka,
159 Japan), EGFR inhibitor (50 μ M) (Cayman), Salirasib (20 μ M) (Focus Biomolecules), and
160 PKC inhibitor (100 μ M) (Santa-Cruz). After incubation for 3 h, 12 h, or 30 h, the follicles
161 were collected and used for experiments. The follicles that successfully ovulated were
162 counted after incubation for 30 h and ovulation rates were calibrated. Medaka rLh was
163 prepared as previously described [37].

164

165 **Cloning**

166

167 As the nucleotide sequence of medaka *epac1* is different from that currently available
168 from the National Center of Biotechnology Information (NCBI) database [43], the gene
169 was subjected to cDNA cloning. The sequence of the 5' unknown region was determined
170 using rapid amplification of 5'-cDNA ends according to previously reported methods [44],
171 except that total RNA purified from preovulatory follicles isolated from ovaries 1 h after
172 ovulation was used. The primers used are given in Supplementary Information
173 (Supplemental Table S1). To confirm the sequence of the full-length cDNA, RT-PCR was
174 conducted using KOD Fx DNA polymerase (Toyobo, Osaka, Japan) with the follicle

175 cDNA. The primers used were Epac1 full-SS and Epac1 full-AS (Supplemental Table S1).
176 The PCR products were phosphorylated, gel-purified, and ligated into a pBluescript SK
177 vector (Agilent Technologies, Santa Clara, CA). The nucleotide sequence of the resulting
178 vector, pBlu-Epac1, was confirmed by sequencing. The determined sequence was
179 deposited into the DDBJ/GenBank/NCBI database [43] (accession number: LC541574).

180

181 **Reverse-transcription (RT) and real-time polymerase chain reaction** 182 **(PCR)**

183

184 Total RNA was purified using Isogen (Nippongene, Tokyo, Japan) according to the
185 manufacturer's instructions. Real-time RT-PCR (qRT-PCR) was conducted according to
186 a method previously described [44]. Ovarian and/or follicular expression of the following
187 gene transcripts were analyzed: three Epac genes (*rapgef3*, *rapgef4a*, and *rapgef4b*), eight
188 Rap genes (*rapgap1a*, *rapgap1b1*, *rapgap1b2*, *rapgap2a1*, *rapgap2a2*, *rapgap2b1*,
189 *rapgap2b2*, and *rapgap2c*), four Pi3k catalytic subunits (*pik3c2a*, *pik3c2b*, *pik3c2g*, and
190 *pik3c3*), four Akt genes (*akt1*, *akt2a*, *akt2b*, and *akt3*), one mTor gene (*mtor*), one Pdk1
191 gene (*pdk1*), and two Creb genes (*creb1* and *creb2*). The primer pairs used are given in
192 Supplementary Information (Supplemental Table S1). For analysis, a KOD SYBR qPCR
193 Mix (Toyobo) or a KAPA Fast qPCR Kit (Nippon Genetics Co., Ltd., Tokyo, Japan) was
194 used. Eukaryotic translation elongation factor 1 alpha (*eef1a1*) was used as the reference
195 gene to normalize the expression level of the target genes.

196

197 **Antibody preparation**

198

199 Recombinant proteins acting as antigens were produced using an *E. coli* expression
200 system. The coding regions of medaka *epac1* and *creb1*, or the partial coding regions of
201 *pik3c2a*, *pik3c2b*, and *pik3c2g* were amplified via PCR with KOD -Plus- Neo DNA
202 polymerase (Toyobo, Tokyo, Japan) using ovary cDNA. The primer pairs used are given
203 in Supplementary Information (Supplemental Table S1). The PCR products were
204 phosphorylated, gel-purified, and ligated into a pET30a vector (Novagen, Madison, WI)
205 previously digested with *EcoRV*. The nucleotide sequence was confirmed by sequencing.
206 Expression and purification of the recombinant proteins [45], immunization of mice with
207 the antigens [37], and antibody purification [46] were performed according to previously
208 described methods. Anti-medaka Akt1 antibody was produced in rabbits by a custom
209 antibody production service (Eurofins Genomics K. K., Tokyo, Japan). The amino acid
210 sequence of the KLH-conjugated peptide used was NH₂-CDSERRPHFPQFSYSAS-
211 COOH. Anti-medaka Mmp15 antibody [36], rat anti-medaka Pgr antibody [34], mouse
212 anti-medaka Pgr antibody [34], and anti-medaka ribosomal protein L7 (Rp17) antibody
213 [42] were prepared as previously described. The other antibodies used were commercially
214 available; Rap1 antibody (GeneTex Inc., Irvine, CA, GTX61875), AKT1 (phospho-
215 Ser473) antibody (GeneTex, GTX61708) and phospho-CREB (S133) (87G3) rabbit mAB
216 (Cell Signaling, Inc., Beverly, MA, 9198S). The specificity of the antibodies was
217 examined by western blot analysis (Supplemental Figure 1).

218

219 **Tissue extract preparation, immunoprecipitation (IP), and western blot** 220 **analysis**

221

222 Preovulatory follicles isolated from medaka ovaries were used directly or after *in vitro*

223 incubation with or without additives for the indicated period of time. Follicles (30 follicles
224 per sample) were sonicated in IP buffer (50 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 0.1%
225 SDS, and 1% Triton X-100) containing 1 × Protease Inhibitor Cocktail (Wako Chemicals,
226 Osaka, Japan) and 1× Phosphatase Inhibitor Cocktail Solution I (Wako) for a few seconds.
227 The samples were then incubated at 4 °C for 2 h with gentle agitation and centrifuged at
228 15,000 × g for 10 min. The resultant supernatants were used for Western blot analysis or
229 IP. Follicle layer extract was prepared as previously described [36]. Protein concentration
230 was determined using a BCA kit (Thermo Fischer Scientific, San Jose, CA).

231

232 IP was performed as previously described [34] with a slight modification. Briefly, the
233 samples (3 mg each) were treated with Protein G-Sepharose (GE Healthcare,
234 Buckinghamshire, England) that had been previously incubated with rat anti-medaka Pgr
235 antibody, anti-medaka Creb1 antibody, anti-medaka Pik3cb antibody, anti-medaka Akt1
236 antibody, normal mouse IgG, normal rabbit IgG, or normal rat IgG. After incubation at 4
237 °C for 16 h, the samples were washed with IP buffer three times, followed by two washes
238 with 50 mM Tris-HCl (pH 8.0). The precipitated materials were boiled in 1× SDS sample
239 buffer for 20 min and used for Western blot analysis. Normal IgGs served as a negative
240 control. The input was loaded with 2% of the extracts used for immunoprecipitation
241 experiments.

242

243 Western blot analysis was performed according to a previous method [36], except that an
244 ImmunoCruzTM IP/WB Optima E System (Santa Cruz, Dallas, Texas) was used as a
245 dilution buffer for secondary antibodies. For detection of phosphoprotein, samples were
246 subjected to SDS-PAGE and transferred to a membrane. The membrane was blocked with

247 a blocking solution (1% BSA, 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, 0.1% Tween 20,
248 0.02% NaN₃) for 1 h at room temperature and incubated with the primary antibody diluted
249 in the wash buffer (10 mM Tris-HCl (pH 7.5), 20 mM NaCl, 0.1% Tween 20) for 1 h.
250 After being washed with wash buffer three times, the membrane was incubated with
251 secondary antibody diluted in an ImmunoCruz™ IP/WB Optima E System for 1 h. After
252 the membrane was washed with the wash buffer three times, signal was detected using an
253 Immobilon Western kit (Millipore, Bedford, MA). Detection of medaka Mmp15 [36] and
254 Pgr [34] proteins was performed as previously described. For detection of Rap1 protein,
255 Western blot analysis was performed according to a previously described method [36].
256 Rpl7 was used as a loading control. Antibody preincubated with the antigen (50 µg) in 50
257 mM Tris-HCl (pH 8.0) for 1 h at room temperature was used as the negative control. The
258 optical band density was measured with CS Analyzer 2.0 Software (ATTO, Tokyo, Japan).

259

260 **Coimmunoprecipitation**

261

262 Coimmunoprecipitation was performed as previously described [36], except that
263 preovulatory follicles 13 and 15 h before ovulation were used for the assay.

264

265 **Preparation of primary granulosa cells (pGC)**

266

267 pGC was prepared as previously described [44] expect that granulosa cells were isolated
268 from preovulatory follicles 14 h before ovulation.

269

270 **Immunohistochemistry**

271

272 Paraffin sections (5 μm thickness) were prepared as previously described [47]. The
273 sections were dewaxed in xylene for 5 min three times, placed in 99% ethanol for 2 min,
274 hydrated in a graded ethanol series, and rinsed in distilled water for 5 min. The specimens
275 were boiled in 10 mM sodium citrate (pH 6.0) for 45 min, washed with PBS for 5 min,
276 and incubated in PBS containing 3% H_2O_2 for 5 min. They were placed in PBS for 5 min
277 and then incubated in 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% Tween, and 1% BSA
278 at room temperature for 60 min. The sections were reacted with anti-medaka Epac1
279 antibody diluted with PBS at room temperature for 60 min, washed with PBS for 20 min
280 three times, and incubated with Dako EnVision+System-HRP Labeled Polymer Anti-
281 mouse (Agilent Technologies, Inc. Santa Clara, CA) diluted with PBS at room
282 temperature for 60 min. After three 20-min washes with PBS, signals were detected using
283 an ImmPACTTM AMEC Red Peroxidase Substrate kit (Vector Laboratories, Burlingame,
284 CA).

285

286 **Detection of active rap1**

287

288 Active medaka Rap1 was detected using a Rap Activation kit (Jena Bioscience, Jena,
289 Germany) according to the manufacturer's instructions. Briefly, preovulatory follicles (30
290 follicles per sample) were punctured with a needle and centrifuged at 1,500 g for 5 min.
291 The pellet was suspended gently in the lysis buffer supplied in the kit. The sample was
292 then centrifuged at 12,000 g for 15 min, and the resultant supernatant was incubated with
293 GST-RalGDS fusion protein and Glutathione-Resin. After 1 h of incubation, proteins
294 coupled with the Resin were recovered according to the method recommended by the

295 manufacturer. Active Rap1 was detected by Western blot analysis using commercially
296 available Rap1 antibody.

297

298 **Chromatin immunoprecipitation (ChIP)**

299

300 ChIP assay was performed as previously described [42], except that preovulatory follicles
301 (100 follicles per group) or follicles (120 follicles per group) cultured *in vitro* with or
302 without an inhibitor were used. Crosslinking, sonication, immunoprecipitation with
303 protein G-Sepharose-coupled medaka anti-Creb antibody, elution, reverse-crosslinking,
304 and RT-PCR were carried out as previously described [42]. Two sets of primers used for
305 PCR are given in Supplementary Information (Supplemental Table S1). Putative CREB
306 binding sites were searched using the free program TFBIND [48]. The site with a high
307 similarity (> 0.9) was selected as the putative binding site.

308

309 **Establishment of a cell line stably expressing medaka Lh receptor (Lhr)** 310 **and Epac1**

311

312 The coding region of Epac1 was amplified using KOD Fx DNA polymerase with the
313 pBlu-Epac1 as a template. The primer pair used was Epac1 pCMV-SS and Epac1 r-AS
314 (Supplemental Table S1). The PCR products were digested with *EcoRI* and *XhoI*, gel-
315 purified, and ligated into a pCMV tag4 vector (Agilent Technologies, Santa Clara, CA)
316 previously digested with the same enzymes. The resulting vector, pCMV-Epac1, was
317 digested with *PciI* (New England BioLabs Inc., Ipswich, MA), and the overhangs were
318 filled in using Klenow Fragment (Takara, Osaka, Japan) to insert a hygromycin gene

319 under control of an SV40 promoter and a poly(A) signal. The gene cassette was amplified
320 via PCR using pGloSensor-22F cAMP Plasmid (Promega, Madison, WI) as a template,
321 KOD-Neo-DNA polymerase (Toyobo) and the primer pair Hyg-SS and Hyg-AS
322 (Supplemental Table S1). The amplified product was phosphorylated, gel-purified, and
323 ligated into the above pCMV-Epac1. The sequence of the resulting vector, pCMV-Epac1-
324 Hyg, was confirmed by sequencing. Construction of pCMV-LHr, which was the vector
325 carrying the medaka *lhr* gene, was performed as previously described [37]. Establishment
326 of a cell line stably expressing medaka Lh receptor (Lhr) and Epac1 was carried out using
327 a medaka caudal fin cell line (OLHNI-2) derived from HNI strain. The cell culture and
328 cell transfection were performed according to previously reported methods [42]. pCMV-
329 LHr was transfected into the cells, and the cells were incubated for 48 h. After incubation,
330 the medium was changed to fresh medium containing 1 mg/ml G-418 (Wako). The cells
331 were cultured for 14 more days, with medium changes every 2 days, followed by isolation
332 and screening of single clones. The resulting cells stably expressing Lhr were further
333 transfected with pCMV-Epac1. The transfection, selection, isolation and screening
334 methods for single clones were the same as those described above, except that 0.5 mg/ml
335 hygromycin B (Wako) was used for selection.

336

337 **Knockout of *akt1* in OLHNI-2 cells expressing Lhr and Epac1**

338

339 A Cas9 nuclease expression vector carrying a hygromycin B resistance gene
340 (pCS2+hSpCas9/Hyg) was prepared as previously described [42]. The sgRNA expression
341 vector was generated according to the previous method [42]. Briefly, the pair of
342 oligonucleotides listed in Supplemental Table S1 was annealed and ligated into pDR274.

343 The resultant vector, pDR274-Akt1 was used for KO experiments. OLHNI-2 cells stably
344 expressing Lhr and Epac1 using ScreenFect A (Wako) were cotransfected with pDR274-
345 Akt1 and pCS2+hSpCas9/Hyg. After incubation for 48 h, the culture medium was
346 exchanged with fresh medium containing 100 µg/ml hygromycin B (Wako), and cells
347 were cultured for another 48 h. The cells were harvested and used for experiments.

348

349 **Luciferase assay**

350

351 A 1,335 or 1,084-bp nucleotide corresponding to a region that included the partial
352 promoter and untranslated region of the *pgr* gene (−1119 to +216, or −868 to +216) was
353 inserted into the pGL3 firefly luciferase expression vector (Promega Corporation,
354 Madison, WI). OLHNI-2 cells stably expressing Lhr and Epac1 were transfected with
355 pGL3-Pgr-1119-bp (−1119 to +216) or pGL3-Pgr-868-bp (−868 to +216) and with pRL,
356 a Renilla luciferase expression vector (Promega Corporation), using ScreenFect A. After
357 the cells were incubated in the presence or absence of rLh for 24 h, luciferase activity in
358 the samples was measured using a Dual-Luciferase Reporter Assay System (Promega
359 Corporation). Firefly luciferase activities were normalized to coexpressed Renilla
360 luciferase activity.

361

362 **Statistical analysis**

363

364 All experiments were repeated 3 to 8 times. Error bars indicate the standard error of the
365 mean (S.E.M.) obtained from 3 to 8 independent experiments. Statistical analysis of all
366 data except for Figures 3E and 4E was conducted using one-way ANOVA followed by

367 Dunnett's post hoc test. Data for Figures 4E were analyzed by one-way ANOVA followed
368 by Tukey's post hoc test, and data for Figure 3E were examined by Student's *t*-test. Equal
369 variation was confirmed using an F-test or Bartlett's test, as appropriate. *P* values less
370 than 0.05 (marked as *) or 0.01 (marked as **) were regarded as statistically significant.
371 For RT-PCR, Western blot analysis, and immunohistochemistry, at least three separate
372 experiments were performed to confirm the reproducibility of the findings, but the result
373 of only one experiment is shown. For qRT-PCR and RT-PCR, cultured follicles (5 follicles
374 per group) or preovulatory follicles (10 follicles per sample) isolated from two fish
375 ovaries were used per experiment.

376

377 **Results**

378

379 ***Pgr* and *Mmp15* expression occur in a PKA-independent manner in** 380 **preovulatory follicles after the LH surge**

381

382 Preovulatory follicles isolated from the fish ovaries 22 h before ovulation were incubated
383 *in vitro* with recombinant medaka Lh (rLh) in the presence or absence of various
384 chemicals (Figure 1A). Follicles incubated with rLh successfully ovulated (Figure 1B).
385 However, rLh-induced follicle ovulation was suppressed significantly when the follicles
386 were incubated in the presence of MDL 12330A (adenylate cyclase inhibitor), H-89 (PKA
387 inhibitor), brefeldin A (EPAC inhibitor), Wortmannin (PI3K/AKT antagonist), AKT
388 inhibitor IV (AKT inhibitor), KG-501 (CREB inhibitor), or GGTI (RAP inhibitor). Next,
389 we examined the expression of *pgr* and *mmp15* in preovulatory follicles or their follicle
390 layers treated with these reagents. Except for H-89, treatment of the follicles with the

391 reagents resulted in significant inhibition of LH-induced *pgr*/Pgr (Figures 1C and 1E) and
392 *mmp15*/Mmp15 expression (Figures 1D and 1F) at both the mRNA and protein level. We
393 further examined the effects of other inhibitors on the expression of *pgr* in preovulatory
394 follicles. rLh-induced follicle expression of *pgr* was suppressed in a concentration-
395 dependent manner when the follicles were incubated in the presence of AC-V (adenylate
396 cyclase inhibitor), Epac-i (EPAC inhibitor), LY (PI3K inhibitor), AKT inhibitor-I (AKT
397 inhibitor), or CTEB-i (CREB inhibitor) (Supplemental Figure S2). The effects of other
398 inhibitors, an EGFR inhibitor, Salirasib (RAS inhibitor), U0126 (MEK inhibitor), and
399 PKC inhibitor, on ovulation and *pgr* expression in the follicles were also examined. These
400 four inhibitors also significantly reduced the rate of follicle ovulation, but none of them
401 showed an inhibitory effect on *pgr* expression in the follicle (Supplemental Figure S3).

402

403 **Epac1-Rap1 plays a role in mediating cAMP signaling to activate the** 404 **downstream pathway in preovulatory follicles after the LH surge**

405

406 We examined whether an exchange protein directly activated by cAMP (Epac) and the
407 downstream effector Rap are involved in the process of *pgr*/Pgr induction. Among the
408 three Epac and eight Rap genes known for medaka (Ensembl genome database,[49]), one
409 mRNA species of *rapgef3* (coding for Epac1) and two mRNA species of *rapgap1a*
410 (coding for Rap1a) and *rapgap1b1* (coding for Rap1b-1) were expressed in large
411 abundance (Supplemental Figure S4) in the ovary 17 h before ovulation, the time when
412 intracellular cAMP increased immediately after the LH surge.

413

414 Epac1 protein was localized in the oocyte cytoplasm of follicles with a diameter less than

415 100 μm , which were in the previtellogenic phase at stage I-III (Figure 2A, arrows). The
416 protein was also expressed in the granulosa cells of follicles larger than 500 μm , which
417 were in a late or post-vitellogenic phase at stage VIII-X (Figures 2A arrowheads and
418 asterisk, 2C and 2D). No signal was observed in the section stained with absorbed
419 antibody, indicating that the signal was specific (Figure 2B).

420

421 We next examined the expression and activation of Rap1a and Rap1b-1 in preovulatory
422 follicles via Western blot analysis. The Rap1 antibody used in this study reacted with both
423 medaka Rap1a and Rap1b-1 (Supplemental Figure S1B and C), and thus, we hereafter
424 refer to the polypeptide detected with this antibody as Rap1 protein. Western blot analysis
425 of the extracts prepared from preovulatory follicles in the first half (-23 to -11 h) of the
426 24 h spawning cycle revealed a single band of approximately 24 kDa (Figure 2E). The
427 polypeptide band was steadily detected at all points of analysis, indicating constitutive
428 expression in the follicles. Active Rap1 was detected using a GST-RalGDS pull-down
429 assay. Active Rap1 was pulled-down by a GST-RalGDS fusion protein using extracts
430 prepared from the preovulatory follicles and precipitated by Glutathione-Resins. The
431 resulting precipitates were analyzed by Western blot using an anti-Rap 1 antibody (Figure
432 2F). Active Rap1 was also detectable at each point of analysis, but the greatest signal was
433 detected significantly around -17 and -15 h (Figure 2G).

434

435 Activated Rap1 was detected in the follicles incubated for 3 h with rLh, but the rLh-
436 induced activation of Rap1 was inhibited by brefeldin A (Figure 2H). Rap1 was also
437 activated when the follicles were incubated with forskolin or 8-Br-cAMP, a cAMP
438 analogue, in the absence of rLh (Figure 2I).

439

440 **Activation of the PI3K-AKT pathway is a downstream event induced by**
441 **Eapc1-Rap1 activated by the LH surge**

442

443 We examined the possibility that the PI3K/AKT pathway may play a role in mediating
444 EPAC1-RAP1 signaling to downstream pathways, eventually leading to *pgr*/Pgr
445 expression.

446

447 PI3K is a heterodimer composed of a regulatory subunit and a catalytic subunit. Medaka
448 possesses four genes for the catalytic subunit and five genes for the regulatory subunit. In
449 this study, we examined the catalytic subunit of Pi3k for its expression in fish ovaries.
450 qRT-PCR showed that three genes (*pik3c2a*, *Pik3c2b*, and *pik3c2g*) were expressed at
451 detectable levels in the ovaries isolated 17 h before ovulation (Supplemental Figure S5),
452 the time when follicles undergo the LH surge. On the other hand, among the four Akt
453 genes (*akt1*, *akt2a*, *akt2b*, and *akt3* in official name) identified for medaka, the expression
454 of *akt1* was the highest among in the -17 h ovary (Supplemental Figure S6).

455

456 Protein expression for the catalytic subunit of three Pi3k was examined by
457 immunoprecipitation/Western blot analysis. Extracts prepared from preovulatory follicles
458 were immunoprecipitated with each antibody and the resulting precipitates were
459 examined by Western blot analysis. The analysis indicated that the Pi3k catalytic subunit,
460 *Pik3c2b* (common name Pi3k-c2 β), was only expressed at detectable levels in
461 preovulatory follicles (Figure 3A, middle panel). We subsequently examined the possible
462 involvement of Pi3k-c2 β in the expression of *pgr*/Pgr. To determine whether Pi3k-c2 β

463 interacts with active Rap1 in follicles that are destined to ovulate, active Rap1 was pulled-
464 down by a GST-RalGDS fusion protein using extracts isolated at various time points in
465 the first half of the 24 h-spawning cycle and precipitated by Glutathione-Resins. The
466 resulting Rap1 fraction was then analyzed by Western blotting using anti-medaka Pi3k-
467 c2 β antibody. A clear band around -15 h of ovulation was detected significantly (Figure
468 3A, upper and lower panel), confirming an interaction between Rap1 and Pi3k-c2 β around
469 this time in preovulatory follicles.

470

471 A steady expression of Akt1 in the preovulatory follicles was shown during the 24 h
472 spawning cycle (Figure 3B). However, an active phosphorylated form (pAkt1) of Akt1
473 was restrictedly detected significantly in the -19, -17 h, -15 h, -13 h, and -3 h-follicles
474 using antibody specific for phosphorylated ⁴⁷³Ser, which is an active mark of Akt
475 activation and is required for its maximal activation [50, 51].

476

477 mTOR and PDK1 are known to be important protein kinases necessary for AKT
478 activation [51]. Constitutive expression of *mtor* and *pdk1* mRNA in the follicles
479 throughout the 24-h spawning cycle was confirmed by qRT-PCR (Supplemental Figure
480 S7), suggesting that Akt1 could be activated in preovulatory follicles.

481

482 Next, the effects of various inhibitors on Akt1 activation were investigated using -22 h-
483 follicles (Figure 3C). Incubation of the follicles with rLh for 3 h rapidly induced pAkt1
484 significantly. The rLh-induced phosphorylation of Akt1 was not inhibited by KG-501 or
485 H-89. However, Akt1 phosphorylation was suppressed by brefeldin A, Wortmannin, and
486 AKT inhibitor IV, suggesting the implication of activated EPAC and PI3K signaling in

487 the generation of pAkt1. To obtain further insights into the role of Akt1, we examined the
488 induction of *pgr*/Pgr expression using a medaka OLHNI-2 cell line stably expressing both
489 the medaka LH receptor (Lhcgr) and Epac1 (Rapgef3). In the cells cultured with medaka
490 rLh, *pgr* expression was upregulated, but this *pgr* expression increase was abolished by
491 the addition of AKT inhibitor IV (Figure 3D). Similar experiments were conducted using
492 a cell line deficient in the *akt1* gene. The Akt1-knockout cells used were confirmed to
493 express little or no Akt1 protein and it was estimated that over 80% of the cells were
494 deficient in *akt1* (Figure 3E). rLh treatment of the control cells induced *pgr* expression,
495 but the same treatment did not induce the expression of *pgr* in the *akt1*-deficient cells
496 (Figure 3F). To confirm the reproducibility of the above finding, we also conducted
497 experiments using single-guide RNA targeting a different *akt1* sequence (data not shown).
498 The results of the two gene knockout approaches were basically the same, suggesting that
499 the system achieved site-specific DNA recognition and cleavage of the target specifically.
500 The results indicate that phosphorylated (active) Akt1 is involved in the expression of *pgr*.

501

502 **Phosphorylated Creb1 functions as a transcription factor for expression** 503 **of the *pgr* gene in preovulatory follicles**

504

505 Medaka has two *creb* genes in the Ensembl genome database: *creb1* and *creb2*. We found
506 that the ovaries of sexually mature medaka exclusively expressed the *creb1* gene
507 (Supplemental Figure S8). Creb1 protein was detected in the preovulatory follicle
508 throughout the 24-h spawning cycle (Figure 4A), but phosphorylated Creb1 was
509 restrictedly detected around -23 h, -15 h, -13 h and -3 h of ovulation (Figure 4B and C).
510 The expression of Pgr in preovulatory follicles was drastically induced at the same timing

511 (-15 h of ovulation) when phosphorylated Creb1 was detected.

512

513 We performed a promoter assay to examine the potential role of Creb1 in *pgr* expression
514 using OLHNI-2 cells stably expressing the medaka Lh receptor (Lhcgr) and Epac1
515 (Rapgef3). The cells were transfected with pGL3-Pgr-1119-bp or pGL3-Pgr -868-bp and
516 then treated with or without rLh. Luciferase activity was significantly increased when the
517 cells transfected with a vector containing the *pgr* promoter and a putative CREB binding
518 site were incubated with rLh (Figure 4D), indicating that the *cre* sequence in the promoter
519 of the medaka *pgr* gene is important for its transcription. Next, Creb1 binding to the *pgr*
520 promoter region was examined with a ChIP assay. Preovulatory follicles isolated at 23,
521 15, 10, and 4 h before ovulation were used for the assay. Creb1 recruitment to the
522 promoter was observed only with the -15 h-follicles (Figure 4E).

523

524 **Akt1 dominantly contributes to the Creb1 activation required for Lh-**
525 **induced follicular *pgr* expression**

526

527 To determine whether Akt1 may be implicated in the Creb1 activation necessary for *pgr*
528 expression in LH-stimulated preovulatory follicles, we first examined the possibility of
529 direct Akt1-Creb1 interaction in the follicle. Materials immunoprecipitated with anti-
530 medaka Akt1 antibody using extracts of the -15 h- and -13 h-follicles contained Creb1
531 (Figure 5A), indicating a direct interaction between Akt1 and Creb1 at the time when
532 activated Akt1 is formed in the follicles. Next, experiments were conducted using the cell
533 line stably expressing Lhr and Epac1 (Control) and the same cell line expressing Lhr and
534 Epac1 but not Akt1 (Akt1-KO). In the control cells, Creb1 was detected irrespective of

535 whether the cells were treated with rLh (Figure 5B, left panel), while active pCreb1(S133)
536 was detected significantly in the rLh-treated cells. pCreb1(S133) was detectable if the
537 control cells were incubated with H-89 or AKT inhibitor IV but was undetectable in cells
538 incubated in the medium containing both H-89 and AKT inhibitor IV. In contrast, the
539 pCreb1(S133) produced in Akt1-KO cells after incubation with rLh completely
540 disappeared after the addition of H-89 in the culture medium (Figure 5B), indicating that
541 the pCreb1(S133) found in Akt1-KO cells was due to the action of Pka. These results
542 indicate that in addition to Pka, medaka Akt1 has the potential to phosphorylate Creb1 in
543 cells, and this finding is consistent with previous reports stating that AKT could be an
544 activator of CREB in mammalian species [52-54].

545

546 We further examined the relative contribution of Akt1 and Pka to the Creb1 activation
547 required for *pgr* expression in Lh-stimulated follicles. When the -22 h-follicles were
548 cultured with rLh, Creb1 was phosphorylated. Neither H-89 nor AKT inhibitor IV alone
549 had an inhibitory effect on rLh-induced Creb1 phosphorylation in the follicles, but the
550 phosphorylation reaction was strongly inhibited by the addition of both inhibitors (Figure
551 5C), which is consistent with the results of experiments using OLHNI2 cells (Figure 5B,
552 left panel). The findings indicate that both Pka and Akt1 contribute to pCreb1 formation
553 in LH-stimulated preovulatory follicles.

554

555 In the -22 h-follicles incubated with rLh for 3 h, pCreb1 was detected (Figure 5D). Further
556 addition of KG-501, an inhibitor that interrupts formation of the CREB functional
557 complex [55], to the culture did not affect Creb1 phosphorylation. ChIP assays were
558 further conducted using primer pair-1 with the preovulatory follicles incubated with rLh

559 for 3 h. The follicles treated with rLh showed Creb1 recruitment to the *pgr* promoter, but
560 its recruitment was strongly inhibited by KG-501 (Figure 5E). In the -22 h-follicles
561 treated with rLh for 3 h, Creb1 binding to the promoter region of the *pgr* gene was
562 observed, but Creb1 binding was significantly reduced by the addition of AKT inhibitor
563 IV to the medium (Figure 5F). The above results confirm that pCreb1 is a transcription
564 factor required for expression of the *pgr* gene. The results also indicate the involvement
565 of Akt1 in Creb1 activation.

566

567 Finally, the localization and expression of the genes and/or proteins that may be involved
568 in the cAMP-EPAC-RAP-PI3k-AKT-CREB signaling pathway for Lh-induced *pgr*/Pgr
569 expression in medaka were examined in preovulatory follicles. Primary granulosa cells
570 isolated from the follicles obtained 14 h prior to ovulation expressed all the components
571 of the signaling pathway (Supplemental Figure S9), indicating that the proteins are
572 spatially and temporally associable in the cells.

573

574 **Discussion**

575 In the present study, many inhibitors were used as blockers of target molecule(s) that were
576 suggested to be involved in the signaling pathway for the induction of medaka Pgr (Figure
577 1C). The outcomes of experiments using inhibitors need to be treated with caution to
578 determine whether they have specific inhibitory activity. In the present study, we found
579 that all inhibitors used inhibited the induction of *pgr* expression in a concentration-
580 dependent manner (Fig. S2). Furthermore, we examined whether the induction was
581 inhibited using two different blockers of cAMP, Epac, Rap, Pi3k, Akt, or Creb, and
582 treatment of preovulatory follicles with all inhibitors caused the inhibition of *pgr*

583 expression. Such a concentration-dependent inhibition and parallelism in the pattern of
584 effects on the follicles strongly argues that all blockers inhibit the target molecule(s).
585 However, our results do not completely rule out the potential toxicity and off-target effects
586 of the blockers at present.

587

588 Our present investigation provides evidence that the
589 cAMP/EPAC/RAP/PI3K/AKT/CREB signaling pathway is activated for LH-induced
590 induction of *pgr*/*Pgr* in preovulatory follicles at ovulation in the teleost medaka (Figure
591 6). Like other teleosts [56], both the theca and granulosa cells of preovulatory follicles
592 express Lh receptors in medaka [37]. However, existing evidence indicates that granulosa
593 cells play a principal role in ovulation with little, if any, contribution of theca cells to the
594 process [57, 58]. Therefore, in this model, the signaling pathway activated immediately
595 after the LH surge in the follicle is illustrated by focusing on the granulosa cell. Lh, which
596 is secreted from the pituitary gland approximately 17 h before ovulation in the 24-h
597 spawning cycle of the fish [37], binds to the Lh receptor expressed on granulosa cells,
598 resulting in synthesis of cAMP through adenylate cyclase activation [33]. The cAMP thus
599 synthesized binds to and activates Epac1. Activated Epac1 then turns on Rap1 by forming
600 the GTP-bound form of Rap1. Active Rap1 then activates the Pi3k-c2b/Akt1 pathway,
601 and the resulting active Akt1 phosphorylates Creb1 to generate the active form of the
602 transcription factor. Active Creb1 is recruited to the promoter region of *pgr* and drives its
603 expression. Judging from the timing of the occurrence of activated Rap1, active Akt1,
604 active Creb1, and active Creb1 binding to the *pgr* promoter region, the pathway becomes
605 active for a duration of only several hours (from -17 to -13 h, with a peak around -15 h)
606 in the granulosa cells of the follicles. This brief window of pathway activation is

607 necessary and sufficient to induce the rapid and transient expression of *pgr*/Pgr that
608 subsequently serves as a regulator of the expression of various ovulation-related
609 genes/proteins. It should be noted that Pgr is a progestin-activated transcription factor and
610 that progestins must be synthesized in the granulosa cells of the follicles in sufficient
611 quantities to activate Pgr. In medaka, 17 α , 20 β -dihydroxy-4-pregnen-3-one (17, 20 β P) is
612 the naturally occurring progestin [56]. Activated Lh receptors on the granulosa cells of
613 preovulatory follicles upon the Lh surge induce a dramatic shift in the steroidogenic
614 pathway from estradiol-17 β to 17,20 β P, resulting in high follicular levels of 17,20 β P [56].
615 Thus, the progestin readily binds to Pgr to induce activation when Pgr is synthesized in
616 the cells.

617

618 We found that Creb1 was phosphorylated by both Akt1 and Pka in the granulosa cells of
619 Lh-stimulated preovulatory follicles. This result indicates that, in addition to
620 cAMP/EPAC/RAP/PI3K/AKT/CREB signaling, the cAMP/PKA/CREB signaling
621 pathway is simultaneously activated in the follicles by the Lh surge. The involvement of
622 cAMP/PKA/CREB signaling in fish ovulation is evident from the current observation that
623 follicle ovulation was almost completely inhibited by H-89. Intriguingly, Akt inhibitor IV
624 strongly inhibited Creb1 binding to the *pgr* promoter region. In addition, all the inhibitors
625 affecting the EPAC/RAP/PI3K/AKT pathway substantially inhibited *pgr*/Pgr expression
626 in the treated follicles. However, the PKA inhibitor H-89 had no significant inhibitory
627 effect on follicular expression of *pgr*/Pgr. These findings indicate that active Creb1
628 generated by Akt1 may be preferably employed for transcription of the *pgr* gene in the
629 follicles (Figure 6). We should note our present observation that neither H-89 nor AKT
630 inhibitor IV alone had an inhibitory effect on rLh-induced Creb1 (Ser133)

631 phosphorylation in the follicles, but the phosphorylation reaction was strongly inhibited
632 by the addition of both inhibitors. We presume that phosphorylated Creb1 could be
633 simultaneously and independently produced through the cAMP/PKA and
634 cAMP/EPAC/RAP/PI3K/AKT signaling pathways in the Lh-stimulated preovulatory
635 follicle and that even one of these pathways was blocked by its respective inhibitor, the
636 other pathway would still be active to produce phosphorylated Creb1. The generation of
637 active Creb1 (Ser133) was completely suppressed only when the two inhibitors were
638 added. Here, we question why Akt1-activated Creb1, but not Pka-activated Creb1,
639 selectively contributes to the subsequent pgr transcription in the cytoplasm of granulosa
640 cells. This could be explained by compartmentalization of signaling molecules and
641 enzymes. Emerging evidence indicates that receptor signaling is restricted to highly
642 organized compartments within the cell and that the functional confinement of signaling
643 pathways provides a mechanism whereby the activation of distinct receptors provokes
644 unique responses [59]. In the same way, signal compartmentalization in granulosa cells
645 could be a cellular determinant that may facilitate the sole involvement of Akt1-activated
646 Creb1 in the expression of pgr/Pgr. Alternatively, beyond the Ser133 of Creb1, an
647 additional amino acid residue site(s) of the protein might be phosphorylated to function
648 as a transcription factor for successful pgr expression, and only Akt1 could also
649 phosphorylate the residue at the additional site. This might allow Akt1-activated Creb1 to
650 participate in the pgr transcription event. In our present study, only the Ser133
651 phosphorylation status of Creb1 was analyzed and we have no information on the
652 phosphorylation at other amino acid residues. Further studies are necessary to determine
653 the exact mechanism by which active Creb1 is generated through the
654 cAMP/EPAC/RAP/PI3K/AKT signaling pathway in the LH-stimulated preovulatory

655 follicle, together with the possibility of Pka involvement in this process. Nevertheless,
656 based on the current data, we tentatively assume that Akt-activated Creb1 mainly
657 participates in *pgr* induction.

658

659 Somewhat surprising is that although *pgr* transcription in the preovulatory follicles
660 appears to occur only for the several hours immediately following the Lh surge (from -17
661 to -13 h), *pgr*/Pgr are detected at high levels for a relatively long period of time (from -
662 15 to -7 h for *pgr* transcript and from -15 to -3 h for Pgr protein) in the 24 h-spawning
663 cycle of the fish [33, 34]. This indicates that rapidly transcribed *pgr* mRNA stably remains
664 in the granulosa cells of preovulatory follicles for a long period, even after the
665 transcription of *pgr* is terminated, ensuring that *pgr* mRNA is translated whenever the
666 transcription factor Pgr may be required for expression of ovulation-related genes. Our
667 previous studies revealed that expression of *ptger4b* (prostaglandin E₂ receptor subtype)
668 [33], *ccni* (cyclin I) [42], *pail* (plasminogen activator inhibitor-1) [47, 58], and *mmp15*
669 [34], which are all ovulation-related genes in medaka, occurs in an Lh- and Pgr-dependent
670 manner. In the expression of these genes, the timing of Pgr binding to the promoter region
671 of the respective genes varies: -12 h for *ptger4b*, -9 h for *ccni*, -6 h for *pail*, and -4 h for
672 *mmp15*. At present, the mechanism by which *pgr* mRNA may be maintained without
673 degradation for a relatively long period of time in the granulosa cells of the follicles
674 undergoing ovulation is not known.

675

676 We believe that this study is the first report elucidating the entire signaling pathway
677 responsible for the expression of *pgr* in preovulatory follicles in vertebrates, especially
678 lower vertebrates, except mammals. Here, a question may be raised as to whether the

679 signaling pathway is unique for fish or conserved among other classes of vertebrates,
680 including mammals. Currently available data suggest the involvement of cAMP, PKA,
681 EGF-like peptides/EGFR, ERK1/2 and IP3/DAG in LH-induced follicular expression of
682 *Pgr*/PGR in mammals [15, 18-23]. Assuming that these signaling mediators play a central
683 role in follicular *Pgr* expression in mammals, the signaling pathway responsible for
684 follicular gene expression is quite different between mammals and medaka. Notably,
685 follicular *pgr* expression was not inhibited by respective inhibitors of the four kinases
686 EGFR, RAS, MEK, and PKC in the fish. This difference may be associated with the
687 distinct ovarian follicle structures. Here, we should note a recent study exploring the
688 signaling pathway associated with *PGR* expression in human uterine endometrial stromal
689 cells [60], which reports that hCG activates the ERK1/2 pathway through EPAC, causing
690 a transient increase in *PGR* transcripts and protein expression.

691

692 cAMP/PKA/CREB signaling also plays a critical role in medaka ovulation, as evidenced
693 by the strong suppression of rLh-induced follicle ovulation by H-89. It was reported in
694 mammals that the expression of some steroidogenic and related genes, such as
695 steroidogenic acute regulatory protein (StAR) and aromatase was regulated via the
696 cAMP/PKA/CREB pathway [61-64]. Iwamatsu and Shibata (2008) showed that forskolin
697 induced 17,20βP production in cultured medaka preovulatory follicles [65]. The findings
698 led the authors to speculate that some steroidogenic genes are regulated via the
699 cAMP/PKA/CREB pathway in medaka ovaries. The PI3K/AKT pathway is suggested to
700 be linked to the activation of RAS [66]. We observed in the present study that rLh-induced
701 follicle ovulation was strongly inhibited by the RAS inhibitor (Figure S3). It may be
702 possible that Ras activated via the Pi3k/Akt pathway is involved in medaka ovulation. In

703 addition, EGFR, RAS, MEK, and PKC protein kinase inhibitors were effective in
704 inhibiting follicle ovulation. All of the above four inhibitors were without effect on LH-
705 induced *pgr*/*Pgr* expression in preovulatory follicles. Future research into the specific
706 roles of the above mediators would contribute greatly to overall understanding of the
707 mechanisms that control ovulation in teleosts.

708

709 In summary, this is the first study in lower vertebrates to report the signal flow from the
710 ovulatory surge of LH to eventual expression of the *pgr* gene in follicles destined for
711 ovulation by demonstrating the role of each signaling component contributing to the
712 pathway.

713

714 **Conflicts of interest:** The authors have no financial conflicts of interest to disclose
715 concerning the study.

716

717 **Author contributions:**

718 K.O. designed the study, performed experiments, acquired and interpreted data and wrote
719 the manuscript. M.H. designed the study, performed experiments, acquired and
720 interpreted data. T.T. designed the study, interpreted data, and wrote the manuscript. All
721 authors reviewed the manuscript and accepted the final version.

722

723

724 **References**

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968

969

970 **Figure legends**

971

972 **Figure 1.** Effects of various inhibitors on the induction of *pgr*/Pgr and *mmp15*/Mmp15
973 expression and ovulation in preovulatory follicles. (A) An outline of preovulatory follicle
974 culture experiments. Follicles isolated from spawning medaka ovaries 22 h before
975 ovulation (-22 h-follicles) were cultured in the presence of recombinant medaka Lh (rLh)
976 with or without various inhibitors. The expected timing of the Lh surge, GVBD, and
977 ovulation *in vivo* and *in vitro* are shown. After 12 h or 30 h of incubation, the follicles or
978 the follicle layers of the follicles were collected for analysis. (B) The -22 h-follicles were

979 cultured in the presence of rLh with the indicated inhibitor and the ovulation rates were
980 determined after 30 h of incubation. ****P<0.01** (ANOVA and Dunnett's post hoc test, N=6).
981 (C and D) The -22 h-follicles were cultured as in (B) for 12 h (for *pgr*) or 30 h (for *mmp15*),
982 and follicular expression of *pgr* (C) and *mmp15* (D) mRNA was determined by qRT-PCR.
983 The expression levels were normalized to those of *eefta* and expressed as the fold-change
984 compared with the levels in follicles cultured without inhibitor (None). ****P<0.01**
985 (ANOVA and Dunnett's post hoc test, N=6). (E and F) The -22 h-follicles were cultured
986 as in (B) for 12 h (for Pgr) or 30 h (for Mmp15), and follicular expression of Pgr (E) and
987 Mmp15 protein (F) was detected by Western blot analysis. The dotted line represents
988 cropping of a single gel. The signal intensity of the Pgr (E) or Mmp15 (F) band was
989 quantified densitometrically, and the ratio of expression of Pgr or Mmp15 to Rpl7 was
990 determined as the relative expression (each lower panel). An asterisk indicates a
991 significant difference at $P < 0.05$ (*) or $P < 0.01$ (**). (one-way ANOVA, post hoc Dunnett test, n =
992 4).
993 4).

994

995 **Figure 2.** Follicular expression of Epac1 and *rap1/Rap1* and *in vitro* Rap1 activation.

996 (A and B) Immunohistochemistry was conducted for the -17 h-ovary using anti-Epac1
997 antibody (A) or anti-Epac1 antibody previously treated with recombinant Epac1 (B).
998 Arrows indicate small follicles less than 100 μm , and arrowheads indicate growing
999 follicles larger than 500 μm . An asterisk indicates a fully grown follicle for ovulation. (C)
1000 The boxed area in (A) is enlarged. (D) The boxed area in (C) is further enlarged. TC, theca
1001 cell; GC, granulosa cell; OM, oocyte membrane. Scale bars indicate 0.5 mm in (A) and
1002 (B), and 0.05 mm in (C). (E) Rap1 was detected in extracts of preovulatory follicles

1003 isolated from ovaries at the indicated time points. As a negative control, the extract of the
1004 -15 h-follicles was processed in parallel using normal mouse IgG. Signals for Rap1
1005 (arrow) and bands corresponding to the antibody used for immunoprecipitation (asterisk)
1006 are shown. (F) Active Rap1 was detected in extracts of preovulatory follicles prepared at
1007 the indicated time points. As a negative control, extract of the -15 h-follicles boiled for
1008 10 min was used. Signals for active Rap1 (arrow) and bands corresponding to the Ral-
1009 GST used for the assay (asterisks) are shown. The dotted line represents cropping of a
1010 single gel. (G) The signal intensity of the active Rap1 band was quantified
1011 densitometrically, and the ratio of expression of active Rap1 to total Rap1 was determined
1012 as the relative expression. An asterisk indicates a significant difference at $P < 0.05$ (*)
1013 compared with the intensity at -23 h. (one-way ANOVA, post hoc Dunnett test, $n = 4$).
1014 (H) The -22 h-follicles were cultured with no additives, with rLh or with both rLh and
1015 brefeldin A for 3 h and then used to detect active Rap1. As a control, total Rap1 protein
1016 was detected using the input fractions. (H) The -22 h-follicles were cultured alone, with
1017 forskolin or with cAMP for 3 h, and then used to detect active Rap1. As a control, total
1018 Rap1 protein was detected using the input fractions. The signal intensity of the active
1019 Rap1 band was quantified densitometrically, and the ratio of expression of active Rap1 to
1020 total Rap1 was determined as the relative expression (each right panel). An asterisk
1021 indicates a significant difference at $P < 0.01$ (**) compared with the intensity in follicles
1022 cultured without rLh (-rLH in (H)) or chemical (None in (I)). (one-way ANOVA, post hoc
1023 Dunnett test, $n = 4$).

1024

1025 **Figure 3.** Follicular expression of Pi3k-c2 β , Akt1, and pAkt1 and the effects of inhibitors
1026 on Akt1 activation. (A) In the upper panel, proteins containing active Rap1 were obtained

1027 through a GST-RalGDS fusion protein and Glutathione-Resin, with follicle extracts and
1028 analyzed by Western blot analysis using anti-medaka Pi3k-c2b antibody. As a negative
1029 control, the extract of -15 h-follicles boiled for 10 min was used. In the middle panel,
1030 Pik3-c2b was detected by immunoprecipitation/Western blot analysis. As a negative
1031 control, the extract of -15 h follicles was immunoprecipitated using normal mouse IgG
1032 bound to protein G-Sepharose. The signal intensity of the Pi3k-c2b band was quantified
1033 densitometrically, and the ratio of expression of Pi3k-c2b (upper panel) to Pi3k-c2b
1034 (middle panel) was determined as the relative expression (lower panel). An asterisk
1035 indicates a significant difference at $P < 0.05$ (*) or $P < 0.01$ (**) compared with the
1036 intensity at -23 h. (one-way ANOVA, post hoc Dunnett test, $n = 4$). (B) pAkt1 (upper
1037 panel) and Akt1 (middle panel) were detected in follicle extracts via
1038 immunoprecipitation/Western blot analysis. As a negative control, the extract of -17 h-
1039 follicles was immunoprecipitated using normal mouse IgG bound to protein G-Sepharose.
1040 The lower panel shows the detection of Rpl7 protein using the input fractions as a control.
1041 The dotted line represents cropping of a single gel. (C) The -22 h-follicles were cultured
1042 in the presence of rLh with inhibitors for 3 h, and the extracts were analyzed for pAkt1
1043 (upper panel) and Akt1 (lower panel) expression using immunoprecipitation/Western blot
1044 analysis. The signal intensity of the pAkt1 band was quantified densitometrically, and the
1045 ratio of expression of pAkt1 to total Akt was determined as the relative expression (each
1046 lower panel). An asterisk indicates a significant difference at $P < 0.05$ (*) or $P < 0.01$ (**)
1047 compared with the intensity at -23 h (B) or in follicles cultured without rLh (Cont in (C)).
1048 (one-way ANOVA, post hoc Dunnett test, $n = 4$). (D) OLHNI-2 cells stably expressing
1049 medaka Lhr and Epac1 were cultured for 12 h in the presence or absence of rLh with or
1050 without AKT inhibitor IV, and the resulting cell extracts were subjected to qRT-PCR for

1051 detection of *pgr* mRNA expression. The expression levels were normalized to those of
1052 *eef1a*. * $P < 0.05$ (ANOVA and Dunnett's post hoc test, N=6). (E) The expression of Akt1
1053 protein in extracts of OLHNI-2 cells stably expressing medaka Lhr and Epac1 (Cont) and
1054 cells deficient for the *akt1* gene (Akt1-KO) was detected by Western blot analysis. Rpl7
1055 was used as a control. The signal intensity of the Akt1 band was quantified
1056 densitometrically, and the ratio of expression of Akt1 to Rpl7 was determined as the
1057 relative expression (right panel). An asterisk indicates a significant difference at $P < 0.01$
1058 (***) compared with the intensity at -23 h (B) or in follicles cultured without rLh (Cont in
1059 (C)). (Student's *t*-test, $n = 4$). (F) The control and Akt1-KO cells in (E) were cultured with
1060 or without rLh. After 12 h of incubation, qRT-PCR detection of *pgr* mRNA was conducted.
1061 The expression levels were normalized to those of *eef1a*. * $P < 0.05$ (ANOVA and Dunnett's
1062 post hoc test, N=6).

1063

1064 **Figure 4.** Detection of Creb1 and pCreb1 in preovulatory follicles and binding of Creb1
1065 to the *pgr* gene promoter. (A and B) Creb1 (A), pCreb1 (upper panel in B) and Pgr (lower
1066 panel in B) were detected in follicle extracts via immunoprecipitation/Western blot
1067 analysis. As a negative control, the extract of -15 h-follicles was immunoprecipitated
1068 using normal mouse IgG bound to protein G-Sepharose. The arrow indicates
1069 Creb1/pCreb1, and the asterisk denotes the band corresponding to the antibody used for
1070 immunoprecipitation. The dotted line represents cropping of a single gel. (C) The signal
1071 intensity of the pCreb1 band was quantified densitometrically, and the ratio of expression
1072 of pCreb1(A) to total Creb1 (B) was determined as the relative expression. An asterisk
1073 indicates a significant difference at $P < 0.05$ (*) or $P < 0.01$ (**) compared with the
1074 intensity at -21 h (one-way ANOVA, post hoc Dunnett test, $n = 4$). (D) A promoter assay

1075 was conducted using OLHNI-2 cells stably expressing medaka Lhr and Epac1. The cells
1076 were transiently cotransfected with pGL3-Pgr-1119-bp with three putative CREB binding
1077 sites or pGL3-Pgr-868-bp without the sites. After 24 h of incubation with or without rLh,
1078 luciferase activity was measured. * $P < 0.05$ (ANOVA and Dunnett's post hoc test, N=6).
1079 UTR, untranslated region; Luc, luciferase. (E) Preovulatory follicles isolated at the
1080 indicated time points were used for a ChIP assay using primer pair-1. As a negative
1081 control, the assay was performed with -15 h-follicles using primer pair-2. The upper panel
1082 illustrates the positions of the two ChIP primer pairs used, the putative CRE response
1083 element, and the TATA box in the upstream region of the transcription start site (indicated
1084 as +1) of the *pgr* gene. ** $P < 0.01$ (ANOVA and Tukey's post hoc test, N=6).

1085

1086 **Figure 5.** Creb1 phosphorylation by Akt1 and its role as a transcription factor in LH-
1087 induced follicular expression of the *pgr* gene. (A) Extracts of -13 h- and -15 h-follicles
1088 (for experiments) and -15 h-follicles (for control) were immunoprecipitated using an Akt1
1089 antibody or normal IgG, and the precipitates were analyzed via Western blot using anti-
1090 Creb1 antibody (upper panel) and anti-Akt1 antibody (lower panel). The signal intensity
1091 of the Creb1 band was quantified densitometrically, and the ratio of expression of Creb1
1092 to Akt1 was determined as the relative expression. (B) OLHNI2 cells stably expressing
1093 medaka Lhr and Epac1 (left panel) and OLHNI2 cells stably expressing medaka Lhr and
1094 Epac1 but lacking medaka Akt1 (right panel) were incubated for 3 h with the inhibitors
1095 indicated. After incubation, pCreb1 (upper panel) and Creb1 (lower panel) were detected
1096 via immunoprecipitation/Western blot analysis. (C) The -22 h-follicles were cultured for
1097 3 h with the inhibitors indicated in the presence or absence of rLh and the resulting cell
1098 extracts were analyzed for pCreb1 (upper panel) and Creb1 (lower panel) expression via

1099 immunoprecipitation/Western blot analysis. The dotted line represents cropping of a
1100 single gel. (D) The -22 h-follicles were cultured for 3 h with or without KG-501 in the
1101 presence or absence of rLh, and the resulting cell extracts were analyzed for pCreb1
1102 (upper panel) and Creb1 (lower panel) expression via immunoprecipitation/Western blot
1103 analysis. As a negative control for immunoprecipitation, the extract of follicles cultured
1104 with rLh for 3 h was immunoprecipitated using normal mouse IgG bound to protein G-
1105 Sepharose. The dotted line represents cropping of a single gel. The signal intensity of the
1106 pCreb1 band was quantified densitometrically, and the ratio of expression of pCreb1 to
1107 total Creb was determined as the relative expression. An asterisk indicates a significant
1108 difference at $P < 0.05$ (*) or $P < 0.01$ (**) compared with the intensity in follicles cultured
1109 without rLh (Cont in (B and C)) or KG-501 (D) (one-way ANOVA, post hoc Dunnett test,
1110 $n = 4$). (E and F) The -22 h-follicles were cultured for 3 h with or without KG-501 (E) or
1111 AKT inhibitor IV (F) and in the presence or absence of rLh. After incubation, a ChIP
1112 assay was conducted using primer pair-1 as in Fig. 4D. $**P < 0.01$ compared with the
1113 enrichments of the follicles cultured without rLh (ANOVA and Dunnett's post hoc test,
1114 $N=6$).

1115

1116 **Figure 6.** A model of the signaling pathway that induces Pgr expression in medaka
1117 preovulatory follicles after the LH surge. For details, see the text.

Figure 1

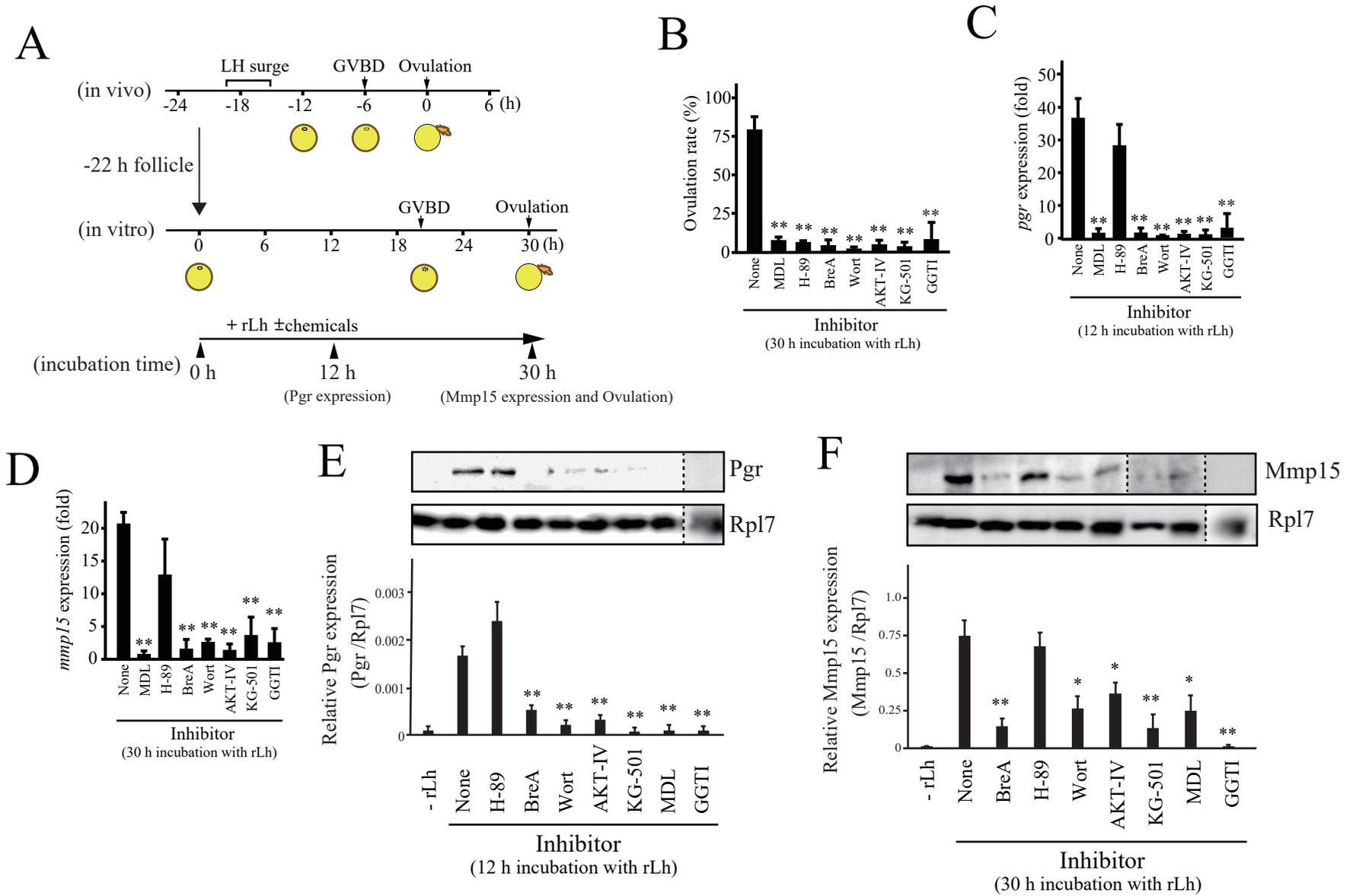


Figure 2

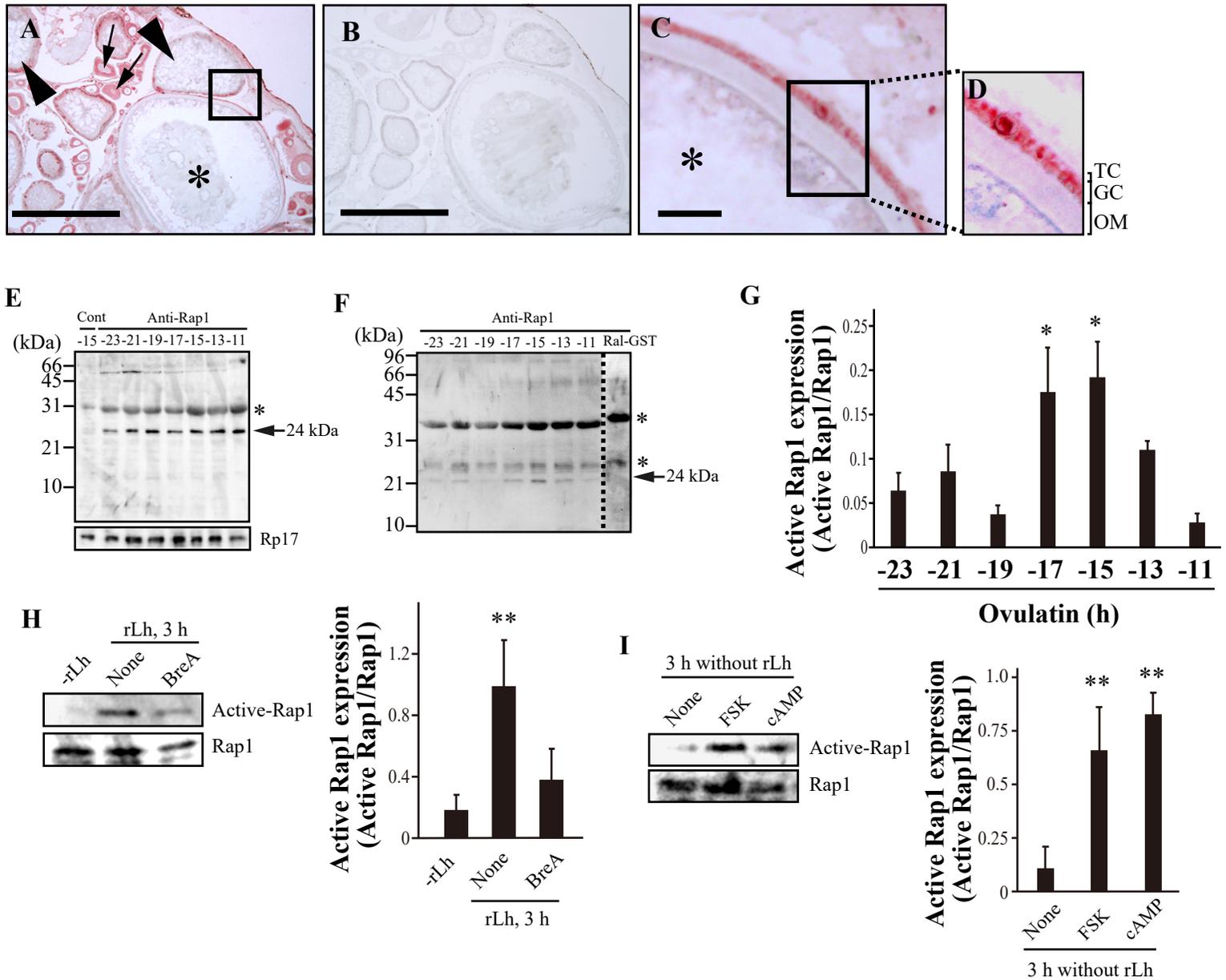


Figure 3

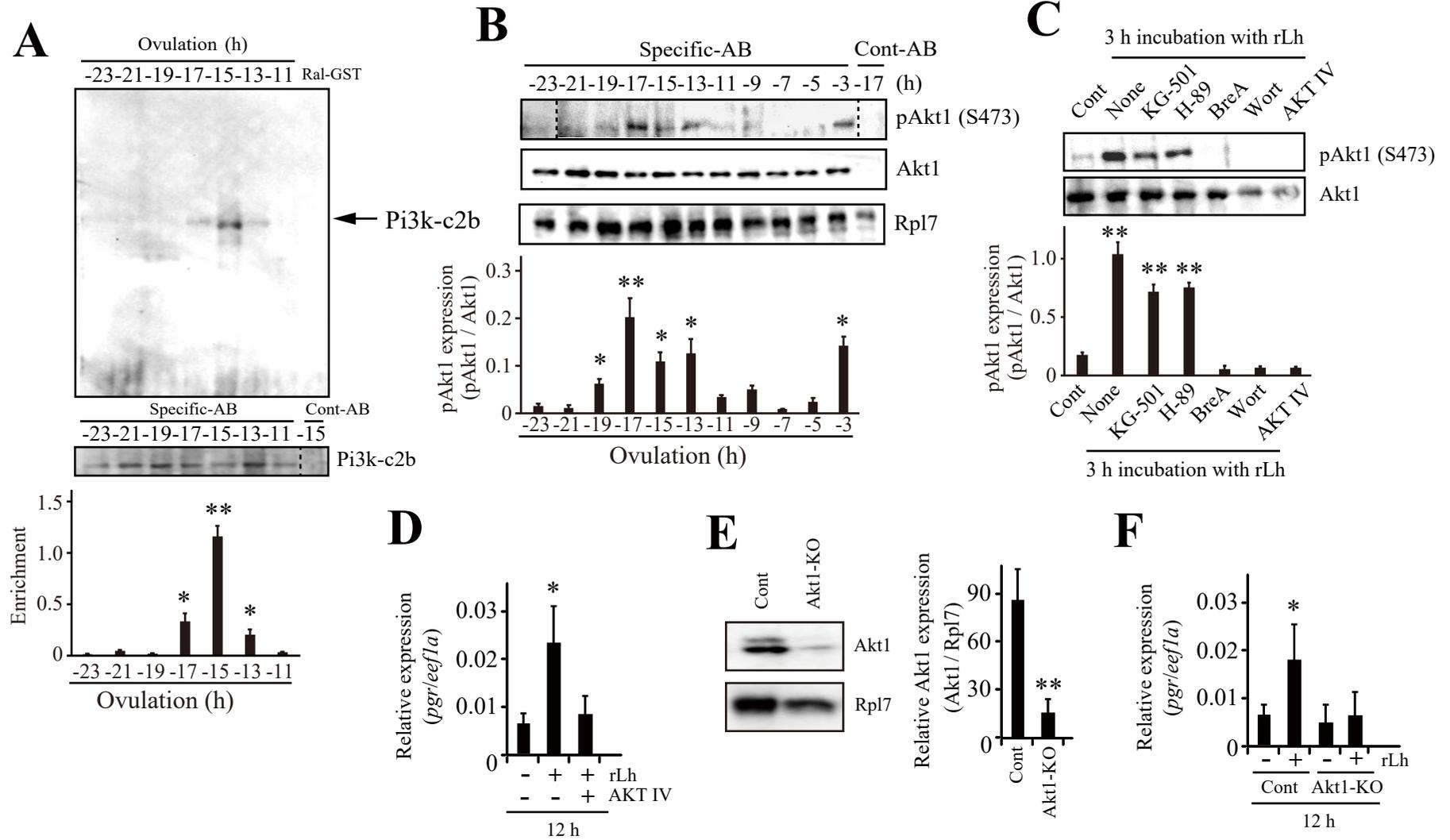


Figure 4

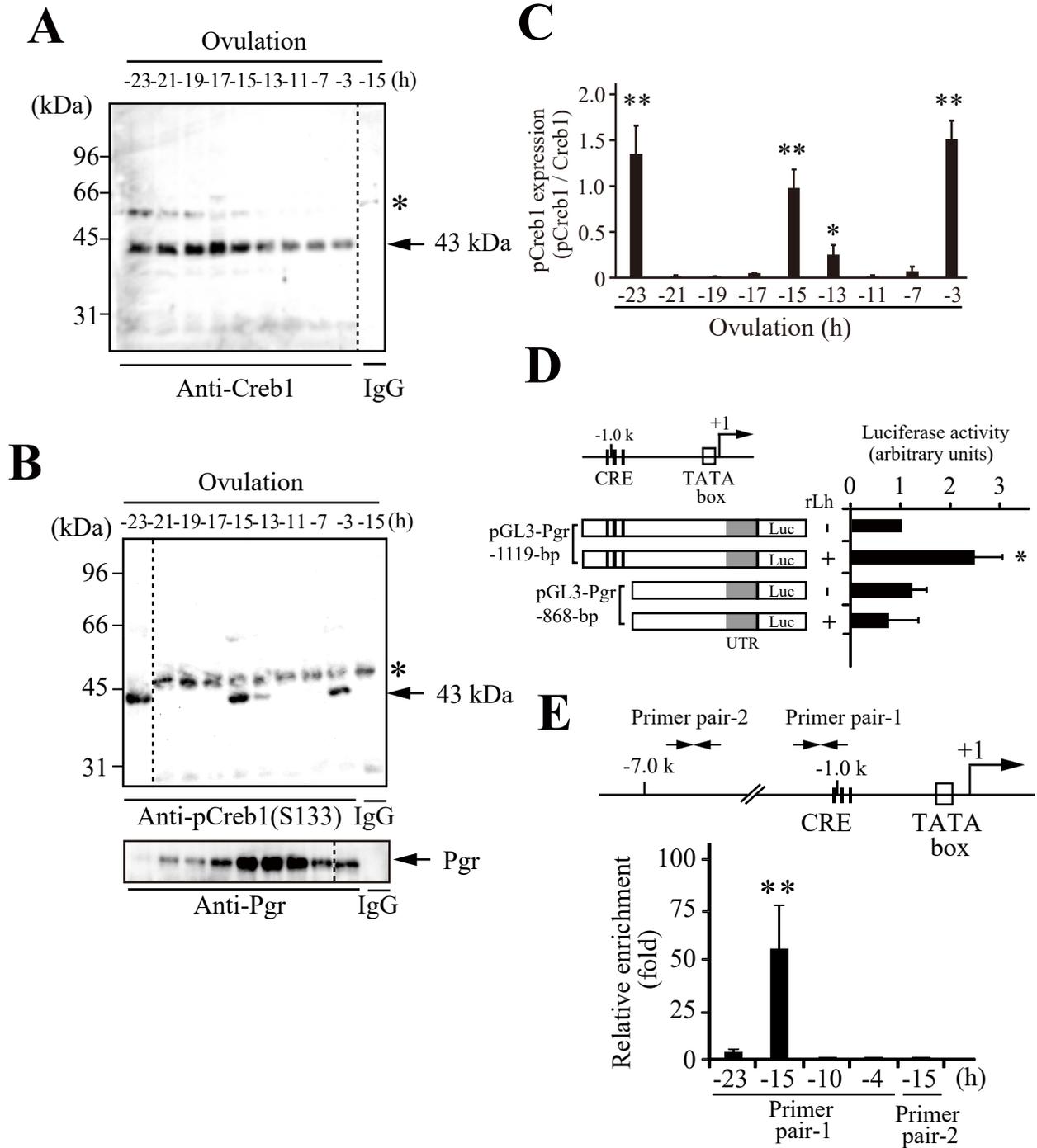


Figure 5

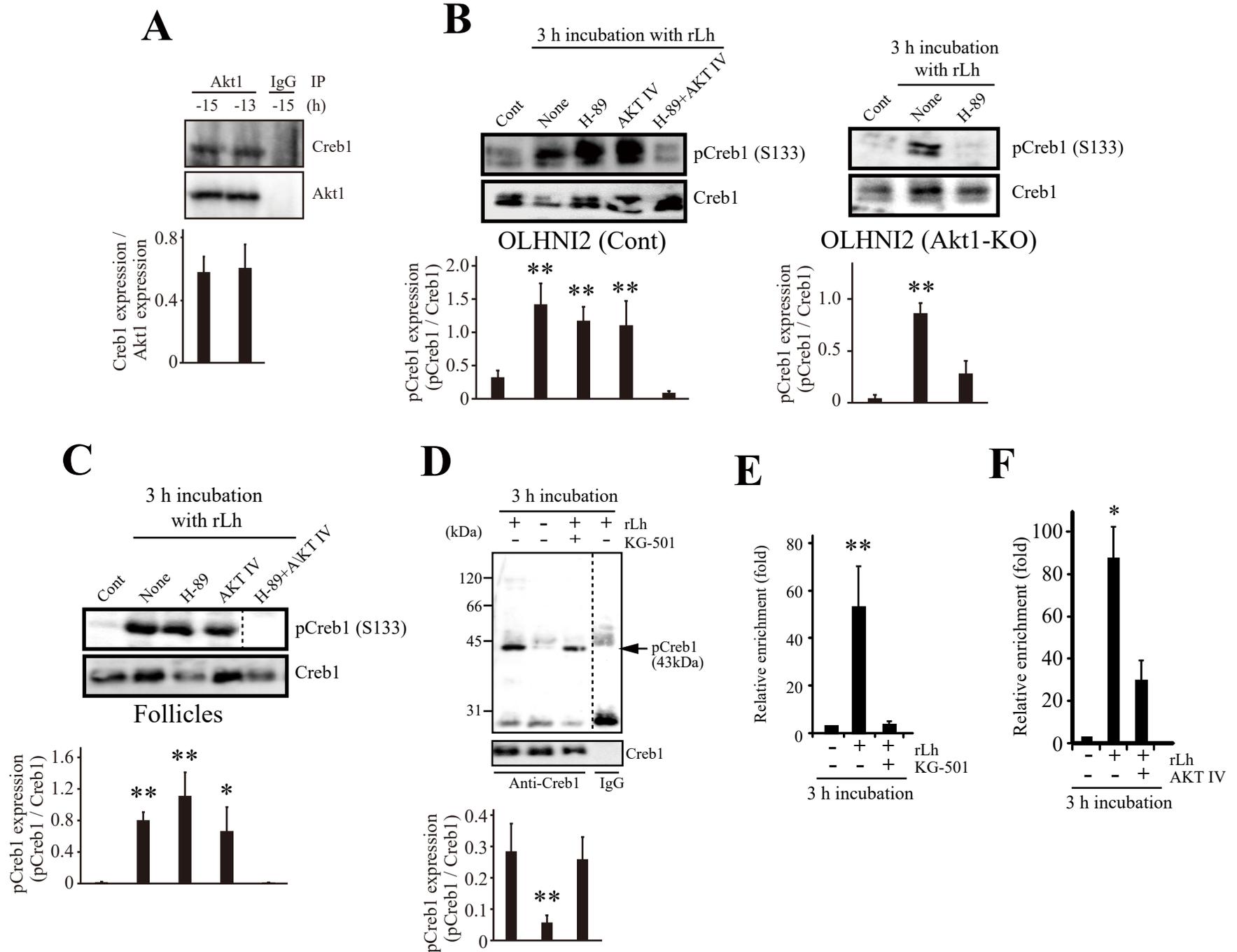
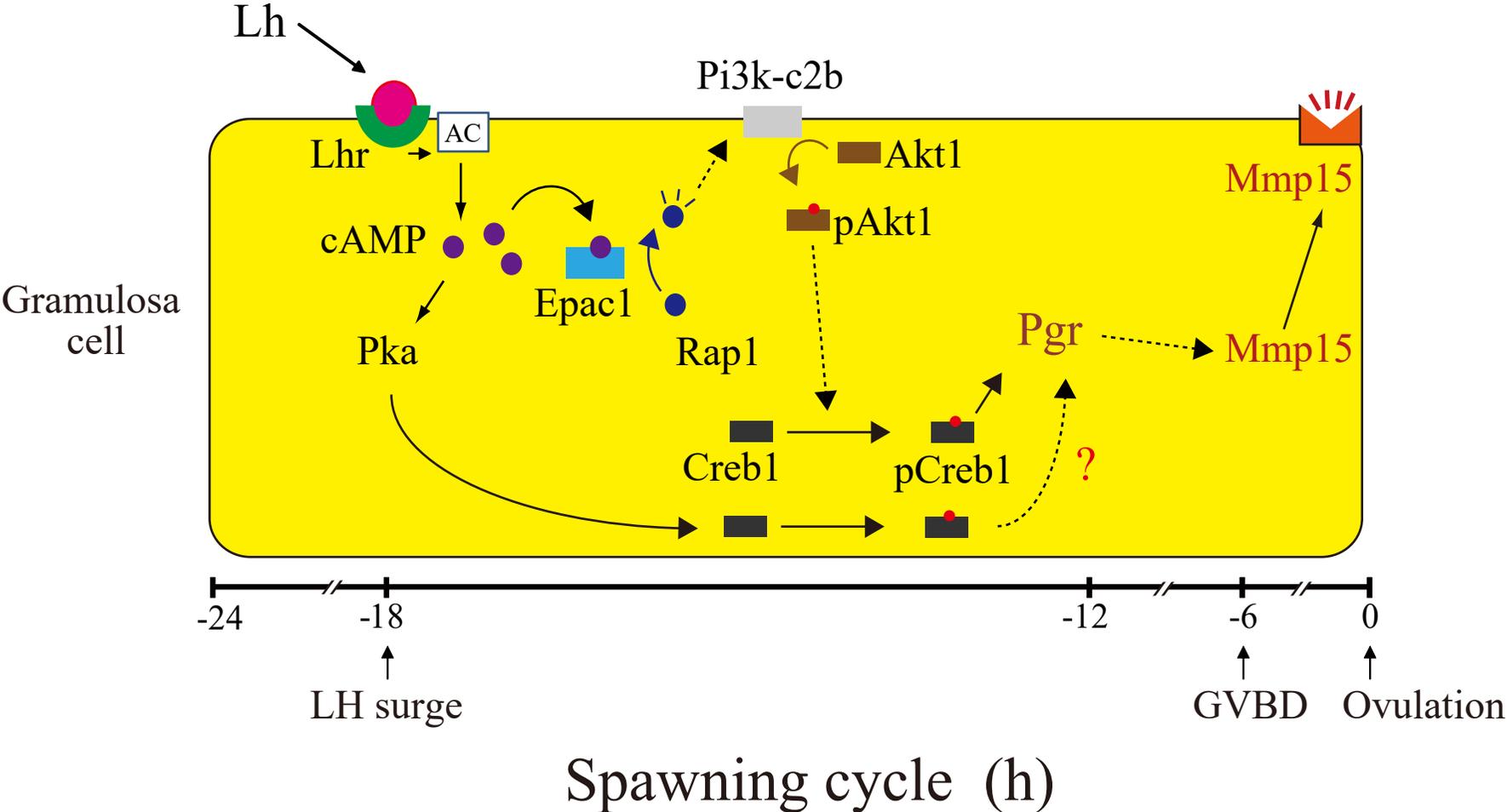
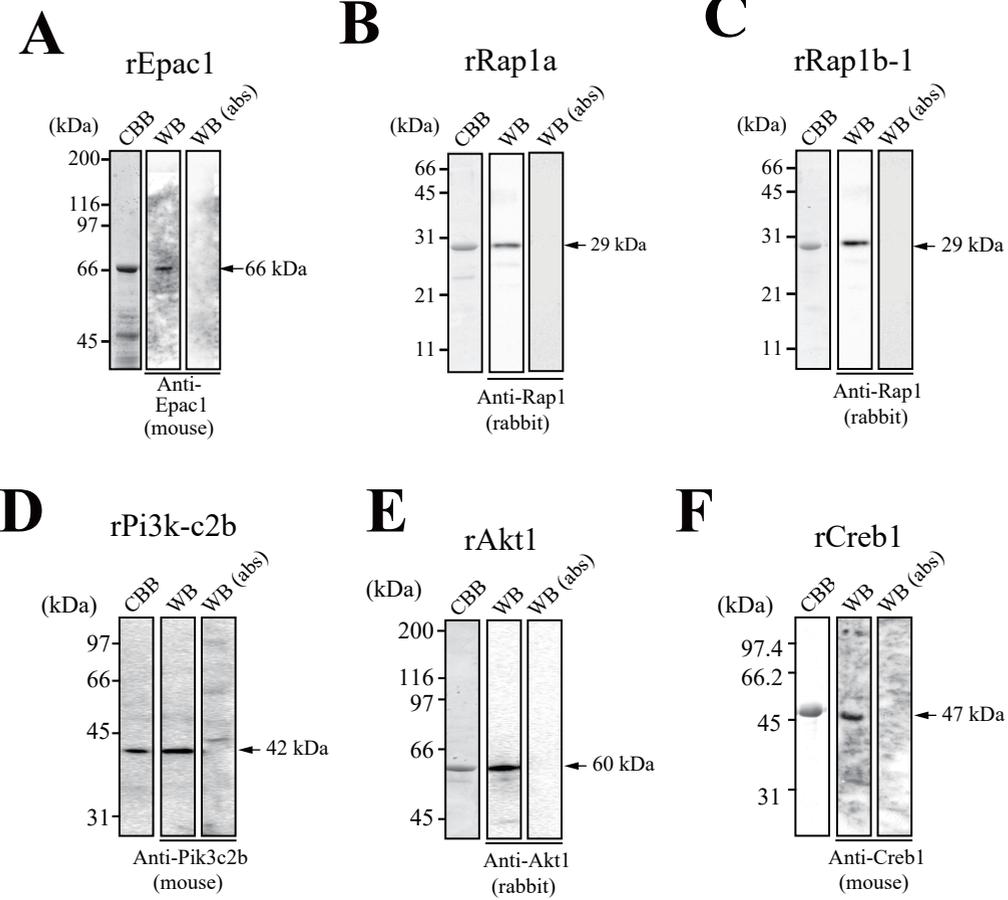


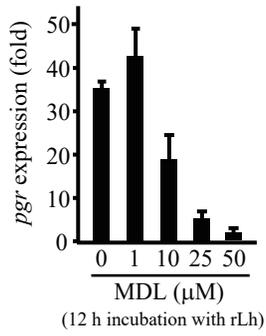
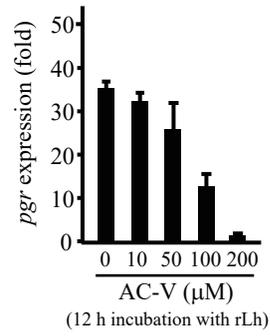
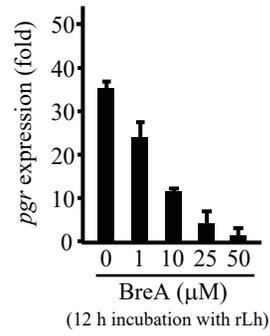
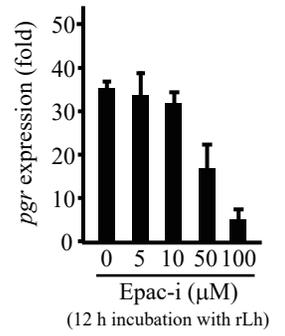
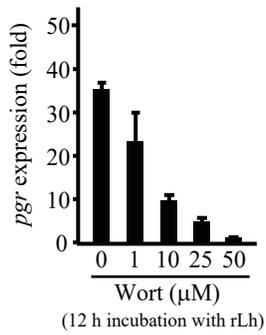
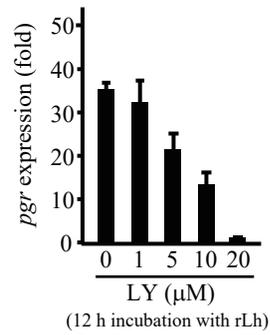
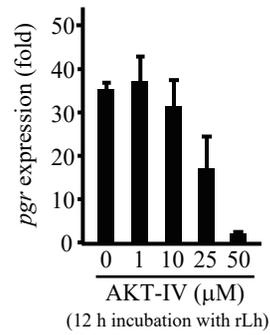
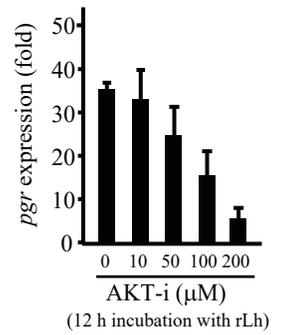
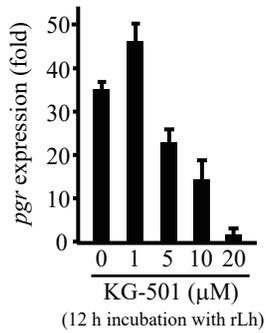
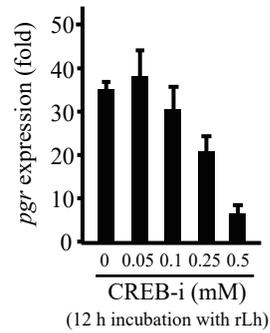
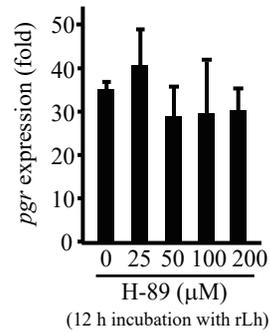
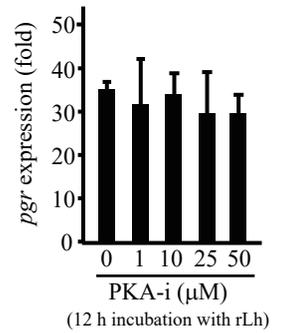
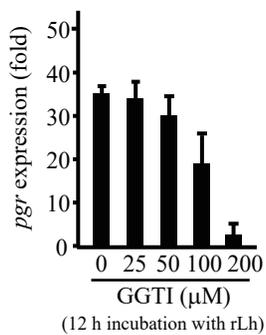
Figure 6



Supplemental Figure S1

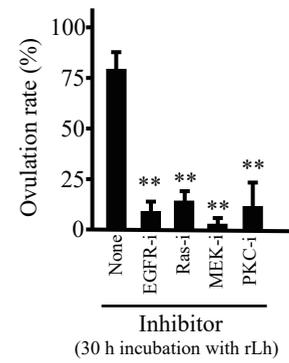


Supplemental Figure S2

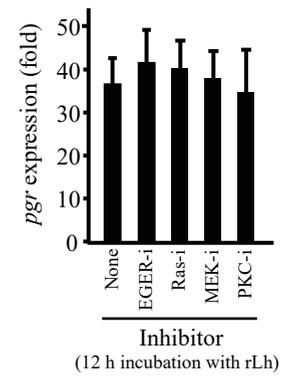
A**B****C****D****E****F****G****H****I****J****K****L****M**

Supplemental Figure S3

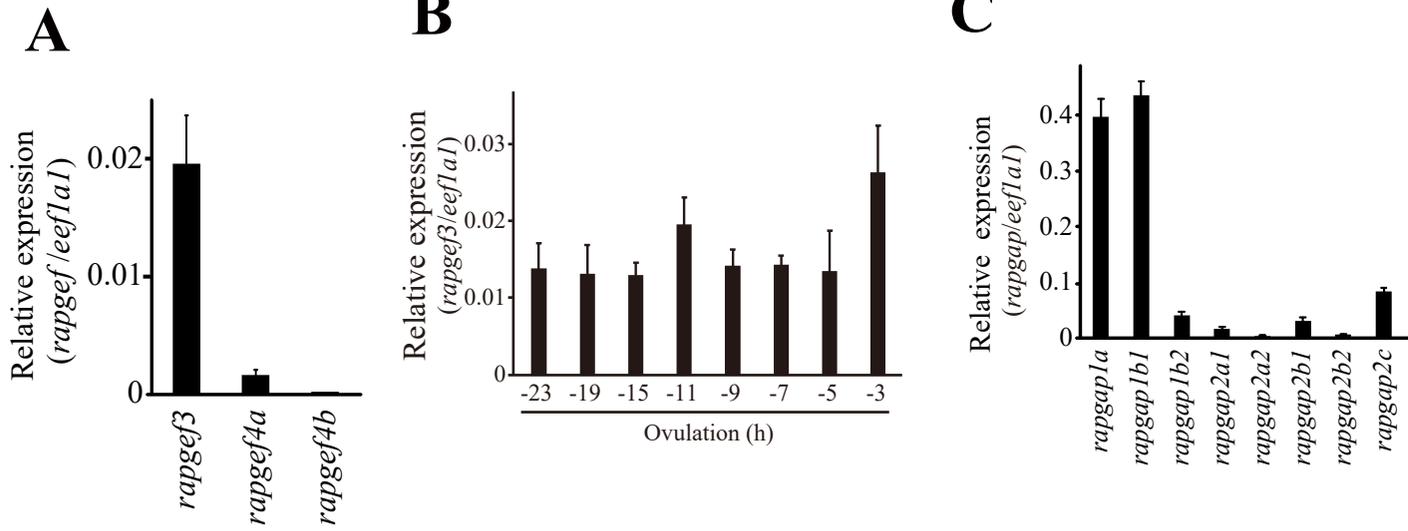
A



B

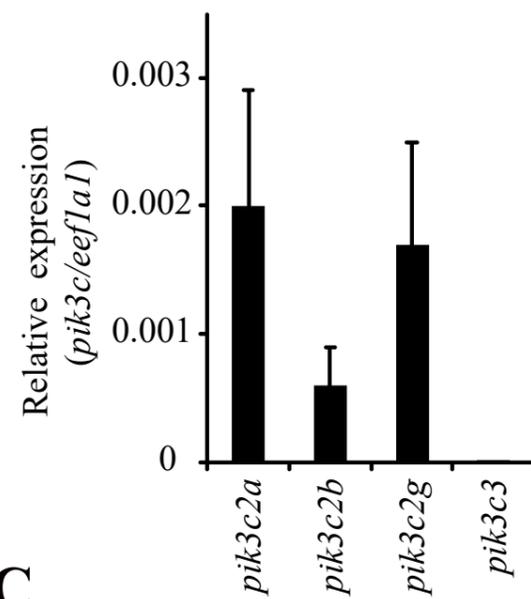


Supplemental Figure S4

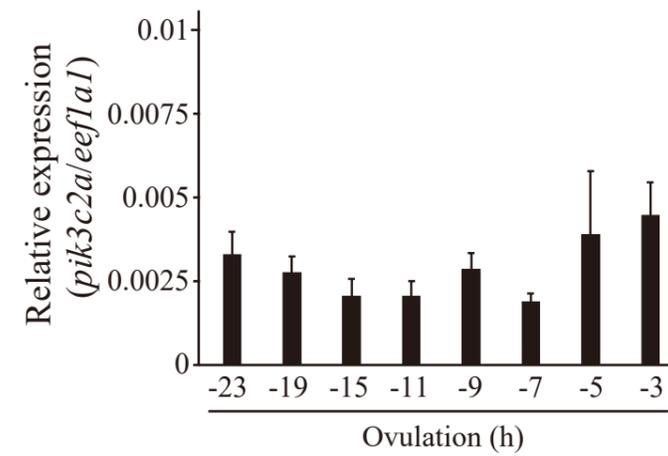


Supplemental Figure S5

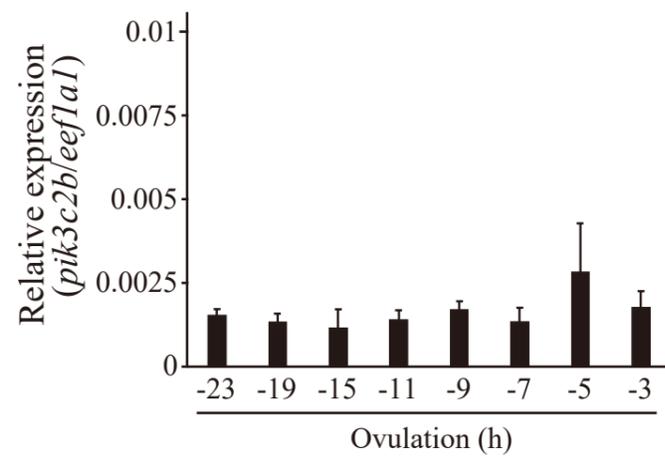
A



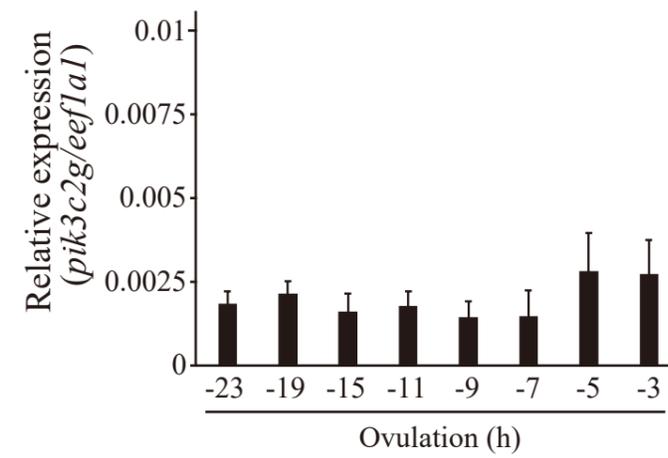
B



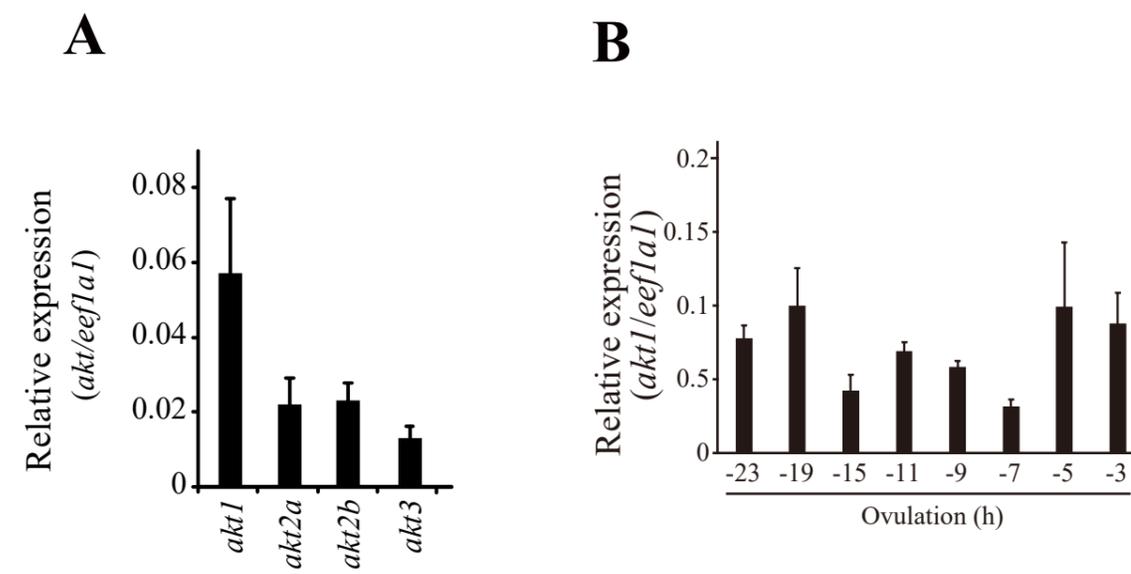
C



D

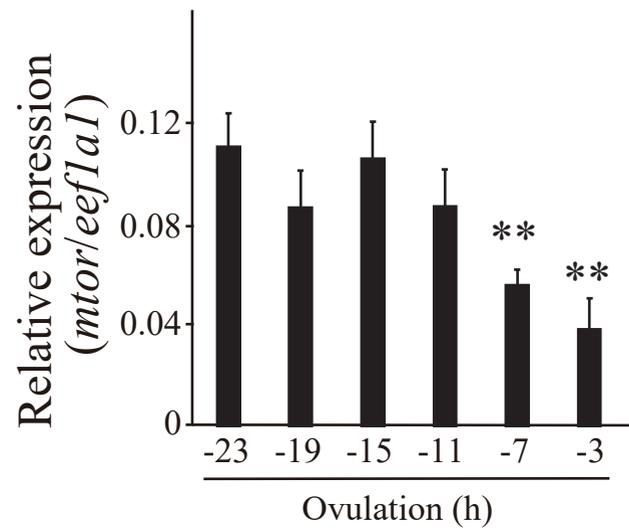


Supplemental Figure S6

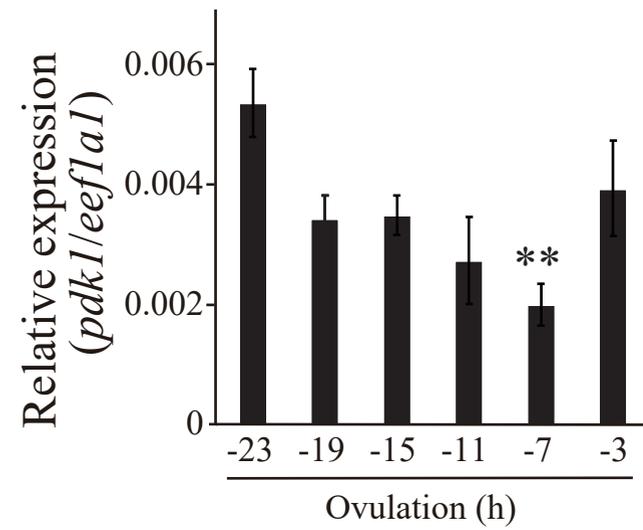


Supplemental Figure S7

A

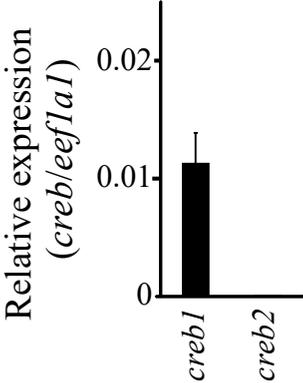


B

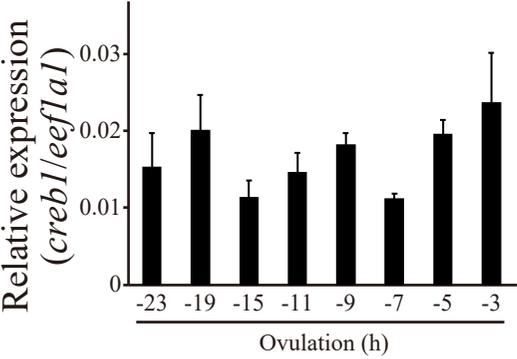


Supplemental Figure S8

A

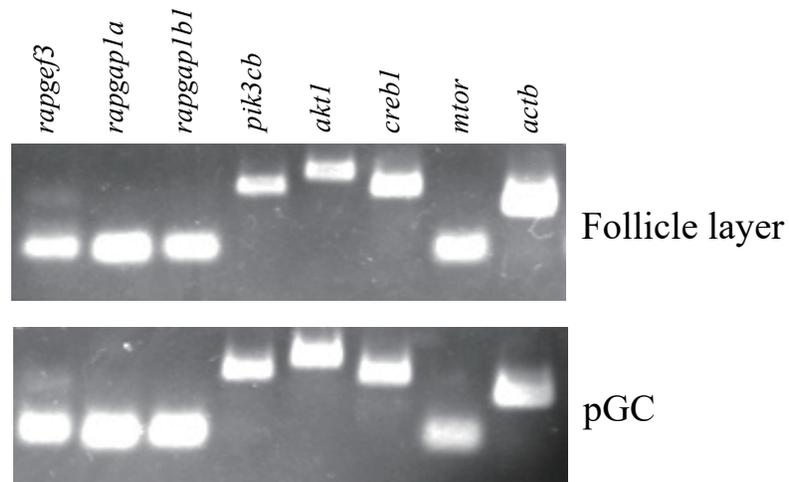


B



Supplemental Figure S9

A



B

