



Title	Involvement of the nuclear progesterin receptor in LH-induced expression of membrane type 2-matrix metalloproteinase required for follicle rupture during ovulation in the medaka, <i>Oryzias latipes</i>
Author(s)	Ogiwara, Katsueki; Takahashi, Takayuki
Citation	Molecular and cellular endocrinology, 450, 54-63 <a href="https://doi.org/10.1016/j.mce.2017.04.016">https://doi.org/10.1016/j.mce.2017.04.016</a>
Issue Date	2017-07-15
Doc URL	<a href="http://hdl.handle.net/2115/68833">http://hdl.handle.net/2115/68833</a>
Rights	© 2017. This manuscript version is made available under the CC-BY-NC-ND 4.0 license <a href="http://creativecommons.org/licenses/by-nc-nd/4.0/">http://creativecommons.org/licenses/by-nc-nd/4.0/</a>
Rights(URL)	<a href="http://creativecommons.org/licenses/by-nc-nd/4.0/">http://creativecommons.org/licenses/by-nc-nd/4.0/</a>
Type	article (author version)
File Information	Ogiwara et al., 2017 MCE.pdf



[Instructions for use](#)

**Involvement of the nuclear progesterin receptor in LH-induced**  
5 **expression of membrane type 2-matrix metalloproteinase required for**  
**follicle rupture during ovulation in the medaka, *Oryzias latipes***

**Katsueki Ogiwara, and Takayuki Takahashi<sup>§</sup>**

10 Laboratory of Reproductive and Developmental Biology, Faculty of Science, Hokkaido  
University, Sapporo 060–0810, Japan

**<sup>§</sup>Corresponding author:**

15 Takayuki Takahashi, Laboratory of Reproductive and Developmental Biology, Faculty  
of Science, Hokkaido University, Sapporo 060–0810, Japan

Tel: 81–11–706–3522

Fax: 81–11–706–4851

E-mail: [ttakaha@sci.hokudai.ac.jp](mailto:ttakaha@sci.hokudai.ac.jp)

20

**Keywords:** medaka, ovulation, LH, Mmp15, nuclear progesterin receptor

## ABSTRACT

Hormonal regulation of the expression of Mmp15, a proteolytic enzyme indispensable  
25 for ovulation in the teleost medaka, was investigated. In an in vitro culture system using  
preovulatory follicles, Mmp15 expression and ovulation were induced in the presence  
of recombinant luteinizing hormone (rLh). Both rLh-induced Mmp15 expression and  
ovulation were  $17\alpha$ ,  $20\beta$ -dihydroxy-4-pregnen-3-one-dependent, suggesting the  
involvement of a nuclear progesterin receptor (Pgr). In vitro follicle ovulation and  
30 Mmp15 expression were reduced by treatment with the Pgr antagonist RU-486. Like  
Pgr, the transcription factor CCAAT/enhancer-binding protein  $\beta$  (Cebpb) was induced  
by rLh. CHIP analyses indicated that Pgr and Cebpb bound to the *mmp15* promoter  
region. These results indicate that the rLh-induced expression of Mmp15 is mediated by  
Pgr and Cebpb. A differential timing of expression of Pgr and Cebpb in the preovulatory  
35 follicles appears to explain the considerably long time-lag from the *pgr* gene activation  
to *mmp15* gene expression.

40

45

## 1. Introduction

Ovulation denotes the shedding of one or more viable oocytes from fully grown ovarian follicles into the reproductive tract and is triggered by the binding of gonadotropin luteinizing hormone (LH) to the receptor in the follicle cells of the follicles destined to ovulate (Richards, 1994; Ma et al., 2004; Espey and Richards, 2006; Lubzens et al., 2010; Zhang et al., 2015; Takahashi et al., 2016). This process is accomplished through a highly regulated cascade of endocrine, morphological, and biochemical events (Espey and Richards, 2006). It is well known that follicle rupture in vertebrate ovulation involves proteolytic degradation at the apical region of ovulating follicles. In the teleost medaka, a variety of proteolytic enzymes are expressed in the follicles (Takahashi et al., 2013). Our previous studies demonstrated that the sequential actions of two distinct proteolytic enzyme systems, the plasminogen activator/plasmin (Plau/plasmin) system and the matrix metalloproteinase (Mmp) system, are required for the hydrolysis of extracellular matrix (ECM) proteins present in the follicle layers of ovulating follicles (Ogiwara et al., 2005; Ogiwara et al., 2012; Ogiwara et al., 2015). In the first step of ECM hydrolysis, active plasmin is produced by the proteolytic processing of liver-derived precursor plasminogen through the follicle-produced active plasminogen activator-1 (Plau1). Plasmin, thus activated, hydrolyzes laminin, a major ECM component constituting the basement membrane (Ogiwara et al., 2012; Ogiwara et al., 2015). As a second step, another proteolytic system involving membrane type 1-Mmp (Mt1-mmp/Mmp14), membrane type 2-Mmp (Mt2-mmp/Mmp15), and gelatinase A (Mmp2) is activated. Mmp2, which is activated by Mmp14 and hydrolyzes type IV collagen, another principal component of the basement membrane, and Mmp15 degrade the type I collagen present in the theca cell layer (Ogiwara et al., 2005). Activation of

the Plau/plasmin system and the Mmp system in the follicle is regulated by plasminogen activator inhibitor-1 (Ogiwara et al., 2015) and the tissue inhibitor of metalloproteinase-2b (Ogiwara et al., 2005), respectively. Interestingly, among the proteolytic enzymes involved in the process, Mmp15 alone was shown to be drastically  
75 induced with recombinant medaka Lh in the granulosa cells of ovulating follicles at the time of ovulation (Ogiwara et al., 2013). This suggests that Mmp15 may be an ovulation-related protein whose expression is under the control of LH in the fish. However, at present, it is not known how the *mmp15* gene expression would be controlled in the granulosa cells of the follicles that have undergone a surge of LH.

80 The aim of the present study was to gain information on the mechanism underlying the LH-induced expression of *mmp15*/Mmp15 in the preovulatory follicles that are destined to ovulate. Our results indicate that the LH-induced expression of the *mmp15* gene is accomplished in two steps: Pgr is first induced by an LH surge, and the resulting Pgr subsequently may serve as a transcription factor for the expression of *mmp15*  
85 mRNA and protein. This study suggests that the transcription factor CCAAT/enhancer-binding protein  $\beta$  (Cebpb) is also involved in *mmp15* gene expression.

## 2. Materials and Methods

### 90 2.1. Animals and tissues

Adult orange-red variant medaka, *Oryzias latipes*, were purchased from a local dealer and used for experiments. Maintenance and acclimation of the fish to the artificial reproductive conditions (14 h light and 10 h dark, 26–27°C) were conducted as previously described (Hagiwara et al., 2014). Under these conditions, the fish ovulated

95 every day around the transition time from the dark to the light period. In the present  
study, ovulation hour 0 was set to the start of the light period. Ovaries, ovarian follicles,  
and follicle layers of the follicles were isolated from the spawning female fish as  
previously described (Ogiwara et al., 2013). Animal cultures and experimentation were  
conducted in accordance with the guidelines for animal experiments of Hokkaido  
100 University and were approved by the Committee of Experimental Plants and Animals,  
Hokkaido University.

## 2.2. In vitro culture of dissected follicles

The culture was performed as previously described (Ogiwara et al., 2013). The outline  
105 of in vitro follicle culture is shown in Fig. 1. Follicles, which had not yet been exposed  
to the in vivo surge of LH, were isolated at 22 h before ovulation from ovaries of the  
fish with established 24-h spawning cycle and were cultured in 4 ml of 90% M199  
medium containing 50  $\mu$ M gentamycin (pH 7.4). The follicles were obtained from two  
to three fish ovaries, pooled, and then divided into control and test groups. The isolated  
110 follicles (approximately 20–25 follicles per group) were cultured with 100  $\mu$ g/ml  
medaka recombinant LH (rLh), 25  $\mu$ M trilostane (TLS, Sigma-Aldrich, St. Louis, MO),  
1  $\mu$ M 17 $\alpha$ , 20 $\beta$ -dihydroxy-4-pregnen-3-one (17, 20 $\beta$ P, Sigma-Aldrich), or 0.1 mM  
RU-486 (also known as mifepristone, Sigma-Aldrich). The duration of incubation and  
the time points when the follicles and/or follicle layers were collected for analysis of the  
115 expression levels of target genes are shown in Fig. 1. The rate of follicle ovulation was  
determined after the follicles were cultured for 30 h. Medaka rLh was produced in  
Chinese hamster ovary k-1 cells as previously described (Hagiwara et al., 2014).

### 2.3. RNA isolation, reverse transcription (RT), and real-time polymerase chain reaction

120 (PCR)

Total RNA isolation, RT, and real-time PCR were conducted as previously described (Fujimori et al., 2011), except that a Thermal Cycler Dice<sup>®</sup> Real-Time II MRQ (Takara Bio. Osaka, Japan) was used for analyses. *Cytoplasmic actin (actb)* was used as a reference gene to normalize the expression of the target genes examined. The primers

125 used in this study are listed in Supplemental Table S1.

### 2.4. Preparation of antigens and antibodies

A 858-bp cDNA coding for the complete Cebpb protein (286 residues) was amplified by RT-PCR using KOD Neo DNA polymerase (Toyobo, Tokyo, Japan) with ovary cDNA.

130 The amplified product was phosphorylated and ligated into the vector pET30a (Novagen, Madison, WI), which had been previously digested by *EcoRV*. The protein antigen was produced in the bacterial expression system. Expression, purification, and dialysis of the protein were performed as previously described (Ogiwara and Takahashi, 2007). Anti-medaka Cebpb antibody was generated using mice according to the method

135 previously described (Ogiwara et al., 2013). Recombinant Pgr was produced as described previously (Hagiwara et al., 2014) and used to immunize rats to obtain anti-medaka Pgr antibody. Mouse anti-medaka Pgr antibody (Hagiwara et al., 2014), rabbit anti-medaka Mmp15 antibody (Ogiwara and Takahashi, 2007), and rabbit anti-medaka Actb antibody (Ogiwara et al., 2012) were prepared as described previously.

140 Antibodies were purified using an Immobilon polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA) membrane as described (Ogiwara et al., 2012), and the resulting purified antibodies were used for the experiments.

## 2.5. Immunoprecipitation

145 Large preovulatory follicles that were isolated from ovaries at various time points or  
follicles that were pre-cultured in vitro with rLh were homogenized in buffer A (50 mM  
Tris-HCl buffer (pH 8.0), 0.15 M NaCl, 1% Triton X-100, and 0.1% SDS) containing  
1×protease inhibitor cocktail (Wako, Osaka, Japan) and 1×phosphatase inhibitor  
cocktail (Wako), and subsequently incubated at 4°C for 2 h with agitation. After  
150 incubation, the follicles were centrifuged at  $15,500 \times g$  for 10 min, and the resultant  
supernatants were used for immunoprecipitation. Immunoprecipitation was performed  
as previously described (Ogiwara et al., 2005) with the following modifications. The  
samples (3 mg each) were treated with Protein G-Sepharose (GE Healthcare,  
Buckinghamshire, England) that had been previously coupled with specific antibodies  
155 or normal IgG. After incubation at 4°C for 16 h, they were washed with buffer A three  
times, followed by 50 mM Tris-HCl (pH 8.0) twice. The precipitant materials were  
boiled in 1×SDS sample buffer for 20 min and used for western blot analyses. The input  
was loaded with 2% of the extracts used for immunoprecipitation experiments. Normal  
IgG served as a negative control. Protein concentrations of the samples were determined  
160 using a Pierce BCA Protein Assay Reagent kit (Thermo Fisher Scientific, San Jose,  
CA).

## 2.6. Western blot analysis

Gel electrophoresis and the transfer to Immobilon PVDF membranes (Millipore) were  
165 performed according to standard procedure. To detect the materials immunoprecipitated  
with rat anti-medaka Pgr antibody, western blot analyses were carried out as follows.



The membranes onto which the proteins were transferred were blocked using Block Ace (Dainippon Sumitomo Seiyaku Inc., Osaka, Japan) at room temperature for 1 h and then reacted with mouse anti-medaka Pgr antibody at room temperature for 1 h. After the  
170 membrane was washed with PBS containing 0.1% Tween-20 four times, it was incubated with an ImmunoCruz™ IP/WB Optima E System (SantaCruz, Dallas, Texas) according to the manufacturer's instructions. Signals were detected using an Immobilon Western kit (Millipore). Actb was detected as a positive control.

#### 175 2.7. 5'-rapid amplification of cDNA end (RACE)

5'-RACE was performed to determine putative transcription start sites for medaka *mmp15* gene. Total RNAs were prepared from ovaries obtained at 3 h before ovulation and were subjected to RT using an Mmp15-GSP-1 primer (Supplemental Table S1). A homopolymeric tail was subsequently added to the 3'-end of the synthesized cDNA and  
180 used as a template for PCR. Two rounds of PCR were carried out using the Mmp15-GSP-2 or Mmp15-GSP-3 primers, and the amplified products were subcloned into a pBluescript II SK (+) and were sequenced. Sequence information for *mmp15* that had been previously submitted to NCBI was updated.

#### 185 2.8. Luciferase assay

A nucleotide corresponding to a region including the partial *mmp15* gene promoter (-948 to -1) was amplified by PCR using KOD Neo DNA polymerase with genomic DNA purified from the liver. The primer pair used were Mmp15-Luc SS and Mmp15-Luc AS (Supplemental Table S1). The PCR product was phosphorylated and  
190 ligated into the pGL3 firefly luciferase expression vector (Promega Corporation,

Madison, Germany), which had been previously digested by *Sma*I. The resultant vector, pGL3-Mmp15, was confirmed by DNA sequencing. The cDNA coding for the complete Cebpb protein was amplified by the RT-PCR described above. The amplified product was phosphorylated and ligated into the vector pCMV tag4, which had been previously  
195 digested by *Eco*RV. The resultant vector, pCMV-Cebpb, was confirmed by DNA sequencing. Luciferase assay, construction of pCMV-Pgr vector, cell culture for 293T cells, and transfection were performed as previously described (Hagiwara et al., 2014). Briefly, pCMV-Pgr, pGL3-Mmp15, pRL mock, and either pCMV tag4 (mock) or pCMV-Cebpb were co-transfected into 293T cells. After 24 h culture, the cells were  
200 added to fresh medium with or without 1 mM 17,20 $\beta$ P and further cultured for 24 h. The cells were then harvested and luciferase activity was measured.

### 2.9. Establishment of a cell line stably expressing medaka Pgr

Cell culture for OLHNI-2 cells and transfection were performed according to the  
205 previous method (Hagiwara et al., 2014). Briefly, pCMV-Pgr vector linearized by digestion with *Pci*I was transfected into medaka OLHNI-2 cells. After 48 h culture, the medium was changed to fresh medium containing 10% FBS and 1 mg/mL G-418 (Wako). The cells were cultured for 20 days with medium changes every 2 days. After the culture, a single colony was isolated and amplified. The expression of *pgr* and Pgr  
210 was confirmed by real-time RT-PCR and western blot analysis, respectively. The cells thus prepared were treated for 24 h with or without 17,20 $\beta$ P (1 mM) and harvested, and the expression of *mmp15* mRNA was examined by real-time RT-PCR.

## 2.10. Chromatin immunoprecipitation (ChIP)

215 ChIP assays were performed as previously described (Hagiwara et al., 2014). Briefly, preovulatory follicles were isolated from the fish ovaries at 4, 10, 15, and 23 h before ovulation. Follicle layer cell fractions obtained from the follicles were fixed and lysed. The fractions were later sonicated and immunoprecipitated with Protein G-Sepharose (GE Healthcare) that had been previously treated with mouse anti-medaka Pgr antibody  
220 or mouse anti-Cebpb. Nonimmune immunoglobulin G was used as a negative control. After immunoprecipitation, precipitates were washed, eluted, and reverse-crosslinked. The extracted DNA was analyzed by real-time PCR. To design PCR primers, a search for the sequence corresponding to the 5'-flanking regions of the *mmp15* and *cebpb* gene was carried out using the Ensembl medaka genome database (Ensembl database). Eight  
225 sets of primers were designed (Supplemental Table S1) and synthesized. As a negative control for real-time PCR, primer pairs were set 6–7 kbp upstream of a putative transcription start site. Putative binding sites of transcription factors were searched for using a free program TFBIND (TFBIND [Internet]; Tsunoda and Takagi, 1999).

## 230 2.11. Statistical analysis

The experiments performed in this study were repeated four to six times, and the results are presented as the mean  $\pm$  S.E.M. Statistical data analyses were performed using a *t-test*, or one-way ANOVA followed by Dunnett's or Tukey's post hoc test, as appropriate. Equal variation was confirmed by F-test or Bartlett's test, as appropriate. P  
235 values less than 0.05 were considered statistically significant. For western blot analyses and immunoprecipitation/western blot analyses, the reproducibility of the results was confirmed after repeating the experiment three to four times, and the results of one

experiment are presented.

### 240 3. Results

#### 3.1. Involvement of 17,20 $\beta$ P in LH-induced expression of Mmp15 in the preovulatory follicle of the medaka ovary

We previously reported that treatment of preovulatory follicles that had not been exposed to LH in vivo with medaka recombinant LH (rLh) in vitro significantly increased the expression of Mmp15 (Ogiwara et al., 2013). Using this experimental system, we confirmed that rLh induces the expression of *mmp15* mRNA in the follicles (Fig. 2A). We further examined the effects of trilostane (TLS), an inhibitor of 3 $\beta$ -hydroxysteroid dehydrogenase (Potts et al., 1978), and 17 $\alpha$ , 20 $\beta$ -dihydroxy-4-pregnen-3-one (17,20 $\beta$ P), the maturation-inducing hormone in the medaka (Sakai et al., 1987), on the follicular expression of the MMP gene and protein. When the follicles were incubated with rLh in the presence of TLS, rLh was not found to induce *mmp15* expression. Further, TLS-suppressed *mmp15* mRNA expression was considerably restored by adding 17,20 $\beta$ P into the culture. The effects of these substances on Mmp15 were examined at the protein level, and the results were consistent with those of *mmp15* mRNA expression (Fig. 2B). TLS treatment drastically inhibited rLh-induced follicle ovulation, while the addition of 17,20 $\beta$ P in the culture could block the ovulation-suppression effect of TLS (Fig. 2C). Following identification of 17,20 $\beta$ P as a factor playing a role in Mmp15 expression in the follicles, we examined whether 17,20 $\beta$ P could directly stimulate the expression of Mmp15. In one experiment, preovulatory follicles, which had not yet been exposed to the in vivo surge of LH, were isolated 22 h before ovulation and then incubated in the presence of both rLh and

11

17,20 $\beta$ P. In another experiment, the follicles, which had been exposed to the in vivo surge of LH, were isolated 12 h before ovulation and were incubated with 17,20 $\beta$ P. No significant change in the temporal Mmp15 expression patterns of the follicles was seen  
265 by externally adding the steroid hormone in both experiments.

Overall, the above results strongly suggest that 17,20 $\beta$ P is involved in the LH-induced expression of Mmp15 in the follicles that are destined to ovulate.

### 3.2. Effect of RU-486 on the expression of Mmp15 in the preovulatory follicle

270 In a preliminary experiment, we found that rLh-induced expression of *mmp15* mRNA and the Mmp15 protein in the preovulatory follicles was inhibited in the follicles co-cultured with RU-486, which was previously shown to act as an antagonist for medaka Pgr (Hagiwara et al., 2014). This implied that Pgr, but not the membrane progesterin receptor, may play a role in the Mmp15 expression in the follicles at ovulation.  
275 Therefore, we further examined whether Pgr contributed to the follicular expression of Mmp15.

Preovulatory follicles isolated 22 h before ovulation were incubated for 30 h in the presence of rLh with or without RU-486. The follicles treated with 10<sup>-4</sup> M RU-486 demonstrated follicle ovulation that was suppressed by 90% (Hagiwara et al., 2014). At  
280 this concentration of the antagonist, the level of rLh-stimulated *mmp15* mRNA expression in the follicles was significantly reduced (Fig. 3A). Interestingly, the reduced follicular expression level of *mmp15* mRNA by RU-486 was restored to that observed with rLh-treated follicles if 17,20 $\beta$ P was added to the culture together with the antagonist. As shown by western blot analysis (Fig. 3B), RU-486 treatment of the  
285 follicles inhibited rLh-stimulated expression of Mmp15, while the inclusion of both  
12

RU-486 and 17,20βP in the culture resulted in expression levels of Mmp15 that were comparable to that of the follicles treated with rLh alone. The effect of RU-486 on the follicular expression of *mmp15*/Mmp15 was examined using the follicles that had been already exposed to the in vivo surge of LH. When the follicles isolated 12 h before  
290 ovulation were incubated for 18 h with RU-486, the expression of both *mmp15* mRNA and protein was significantly reduced (Fig. 4); these results were consistent with those obtained of follicles treated in vitro with both rLh and RU-486 (Fig. 3A).

Next, the effect of RU-486 treatment of the preovulatory follicles on the expression of Pgr was examined. The in vivo expression of the Pgr protein in the preovulatory  
295 follicles was increased at 15 h before ovulation and sustained thereafter (Fig. 5A). The follicles, which were isolated from the fish ovary at 22 h before ovulation, were cultured in vitro in the presence of rLh. Under this condition, Pgr protein levels became detectable around 9 h after the start of incubation (Fig. 5B), confirming our previous results (Hagiwara et al., 2014). Neither the *pgr* transcript level nor the protein level  
300 were affected by the addition of RU-486 (Fig. 5C and 5D), indicating that RU-486 had no effect on the Lh-induced expression of Pgr itself.

The above results are indicative of participation of Pgr in the expression of Mmp15 in the follicles undergoing ovulation.

### 305 3.3. Binding of Pgr to the promoter region of the *mmp15* gene

To examine whether Pgr would be involved in the expression of the *mmp15* gene in the preovulatory follicles, ChIP assays were performed using the Pgr antibody. We searched putative binding sites of Pgr and found nucleotide sequences showing partial homology to progesterone response element (PRE) at eight sites in the 1.5 kb upstream region of  
13

310 the transcription site of the *mmp15* gene. In the assay, we initially used eight primer  
pairs, seven of which were set in the above 1.5 kb promoter region at intervals of  
approximately 30–170 bp to amplify the regions including each of the Pgr binding sites.  
Among the eight primer pairs used (Fig. 6A), primer pair-1 and pair-2 were successful  
at amplifying an expected 101 and 210 bp nucleotide, respectively, when preovulatory  
315 follicles were isolated from the fish ovary 4 h before ovulation (Fig. 6B). Preovulatory  
follicles isolated 23, 15, 10, and 4 h before ovulation were used for the ChIP  
experiments using primer pair-1. Pgr recruitment to the promoter was observed for the  
follicles isolated 4 h before ovulation (Fig. 6C). We further examined the effect of Pgr  
on the expression of *mmp15* using OLHNI-2 cells, a cell line established using cells that  
320 originated from the medaka fin. When the cells stably expressing Pgr were treated with  
17,20βP for 24 h, a significant increase in *mmp15* expression was observed (Fig. 6D),  
which suggested that the Pgr-mediated transcription of the medaka *mmp15* gene is  
dependent on the ligand 17,20βP. The above results indicate that Pgr is involved in the  
expression of *mmp15*/Mmp15 in the follicles undergoing ovulation.

325

#### 3.4. Requirement of CCAAT/enhancer-binding protein β (Cebpb) in the expression of *mmp15*/Mmp15

Knowing that the *mmp15* gene expression may be under the control of the transcription  
factor Pgr in the ovulating follicle, an important question arises on the timing of the  
330 expression of these two genes. The expression of *pgr* mRNA peaks at 11 h before  
ovulation (Hagiwara et al., 2014), while *mmp15* mRNA becomes detectable at 2 h  
before ovulation (Ogiwara et al., 2005). We therefore conducted experiments to gain  
insights into the mechanism by which the onset of transcription of the *mmp15* gene

takes time as long as approximately 9 h after *pgr* gene expression. We hypothesized that, in addition to Pgr, some other transcription factor(s) may play a role for the expression of *mmp15* gene in the follicle, and that the 9-h time lag may represent the time required for the induction of such a factor(s). In our attempts to search transcription factors that might be involved in the *mmp15* gene expression, we found that *cebpb* mRNA expression increased in the preovulatory follicles in association with ovulation. The time course of *cebpb* mRNA levels was investigated using the preovulatory follicles isolated from the fish ovaries at various time points of the 24-h spawning cycle. Levels of *cebpb* mRNA remained low between 23 h and 15 h before ovulation, but *cebpb* expression levels increased as ovulation approached (Fig. 7A). When preovulatory follicles isolated from the fish ovaries 22 h before ovulation were cultured with rLh, levels of *cebpb* mRNA increased (Fig. 7B). To detect Cebpb protein in the follicle, a mouse anti-medaka Cebpb antibody was raised in this study. Recombinant medaka Cebpb (rCebpb) was prepared in the *E. coli* expression system (Fig. 7C, left panel). rCebpb was used as an antigen on immunization of the animal. The antibody recognized the antigen, but the antibody previously treated with rCebpb no longer detected the antigen (Fig. 7C, right panel), indicating that our Cebpb antibody was specific. Western blot analysis showed a single band at a position of approximately 43 kDa with the extracts of follicle layers of preovulatory follicles isolated between -7 h and -3 h of ovulation (Fig. 7D); these results were consistent with those of *cebpb* mRNA (Fig. 7A).

We next examined the possibility that Cebpb directly binds to the promoter region of the medaka *mmp15* gene. We found numerous putative binding sites of Cebpb in the 2.0 kb upstream region of the transcription site of the *mmp15* gene (Fig. 6A). CHIP assays were performed using a specific antibody for medaka Cebpb with preovulatory

15



follicles isolated 4 h before ovulation in a way similar to the ChIP experiment for Pgr. Significant enrichment was observed with primer pair-1 and pair-7 (Fig. 8A). ChIP  
360 assays were further conducted with the preovulatory follicles isolated 10 h and 4 h before ovulation using primer pair-1, primer pair-7, and primer pair-8 (control). The expected nucleotide fragment was amplified in follicles obtained 4 h before ovulation with primer pair-1 and primer pair-7 (Fig. 8B). We further examined the effects of Pgr and Cebpb on *mmp15* gene expression by luciferase assay using HEK293T cells. The  
365 cells were simultaneously transfected with three expression vectors (pCMV-Pgr, pGL-Mmp15, and pRL) or four expression vectors (pCMV-Pgr, pGL-Mmp15, pRL, and pCMV-Cebpb), and were treated with or without 17,20βP. A subtle, but significant, increase in luciferase activity occurred in the cells expressing Pgr when the cells were treated with 17,20βP (Fig. 8C). Further increase in the luciferase activity was seen if the  
370 cells expressing both Pgr and Cebpb were incubated with 17,20βP.

Finally, we examined the possibility that rLh-induced expression of the *cebpb* gene in the preovulatory follicle might be under the control of Pgr, whose expression was also LH-inducible and preceded the *cebpb* expression. To this end, preovulatory follicles were isolated 22 h before ovulation and were further incubated for 25 h with rLh in the  
375 presence or absence of Pgr antagonist RU-486 ( $10^{-4}$  M). No detectable change in the levels of *cebpb* mRNA expression was observed in the treated follicles (data not shown). Furthermore, ChIP assays were performed using Pgr antibody with preovulatory follicles isolated 4 h before ovulation. PCR using the precipitated materials with primer sets that were designed and synthesized according to the nucleotide sequence  
380 corresponding to the 5'-flanking region of the medaka *cebpb* gene (Ensembl database), did not yield any amplified fragment (data not shown). The results suggest that the

induced expression of the *cebpb* gene occurred in a Pgr-independent manner.

The above results suggest that *mmp15* expression is regulated by both 17,20 $\beta$ P-associated Pgr and Cebpb.

385

#### 4. Discussion

In the present study, an in vivo interaction of Pgr with the *mmp15* gene promoter was experimentally demonstrated. None of the eight PRE-like sequences found in the 5' upstream region appeared to yield a palindromic sequence that could form a hairpin-like structure. No potential PRE half-site (5'-TGTTCT-3') was found in the region either. These considerations tempt us to speculate that Pgr may not bind to the 5' upstream region of the medaka *mmp15* gene. Previous studies indicated that complexed Pgr could be tethered indirectly to other transcription factors that bind to the DNA binding sites on promoters of progesterone-regulated genes (Jacobsen and Horwitz, 2012). Taking these into consideration, we tentatively speculate that Pgr-dependent expression of the *mmp15* gene in the medaka preovulatory follicles with impending ovulation may result from an intimate interaction between Pgr and other key transcription factor(s) in the promoter region of the gene. In this process, Pgr may act as an inducible co-regulator for the factors, which has been previously proposed as the role of Pgr in Pgr-regulated gene expression in mammalian ovaries (Robker et al., 2009). To establish the validity of this assumption, further studies are required, including identification of the key factor(s) that interact with Pgr. This study is now underway in our laboratory.

As revealed by ChIP analysis, the binding of Cebpb could occur at two distinct sites in the promoter region of the *mmp15* gene. These two regions contain several putative Cebpb binding sites. Precise sites for Cebpb binding are unknown at present. Interesting

is the recruitment of both Pgr and Cebpb to the region immediately upstream of the transcription start site of the *mmp15* gene. One may ask whether Cebpb could be a factor interacting with Pgr upon transcription of the medaka *mmp15* gene in the follicles. Indeed, we found that these two transcription factors interacted with each other, as  
410 examined by immunoprecipitation/western blot analysis using follicles isolated 4 h before ovulation (our unpublished observation). This observation argues for the idea that Cebpb might have a role in Pgr-dependent expression of the *mmp15* gene by interacting with Pgr. Further work is warranted to clarify the precise mechanism involving Pgr and Cebpb in the follicular expression of the *mmp15* gene.

415 The fact that the involvement of both Pgr and Cebpb in the expression of *mmp15* gene in the preovulatory follicles that are destined to ovulate may explain, in part, the considerably long time-lag from the *pgr* gene activation to Pgr-regulated *mmp15* gene expression. Our tentative explanation for delayed appearance of *mmp15* transcripts after *pgr* expression is as follows: in the follicles undergoing ovulation, Pgr is induced in the  
420 follicle cells of the follicles after LH surge by 11 h before ovulation in 24-h spawning cycle of the fish (Hagiwara et al., 2014). By that time, the synthesizing system for the Pgr agonist 17,20 $\beta$ P is activated, and thus, follicular levels of the progestin are high enough to form 17,20 $\beta$ -associated, active transcription factor Pgr. Until 7 h before ovulation, the *mmp15* gene transcription would not occur in the follicle cells even  
425 though active Pgr may be available. Around 7 h before ovulation, another transcription factor Cebpb would be induced in the follicle cells as a result of ovulatory LH surge. The availability of Cebpb, together with active Pgr, would allow the cells to transcribe the *mmp15* gene. It should be noted that there was still 5 h by the onset of *mmp15* transcription in vivo. This may indicate that further unknown factors are required for the  
18

430 gene expression. To decipher the detailed mechanism of *mmp15* expression in the  
follicles, further studies, including the identification of other indispensable factors, are  
necessary. Nevertheless, the present finding of the involvement of *Cebpb* in the medaka  
*mmp15* gene expression gives a clue for answering a question of why *mmp15*  
transcription takes a considerable time after *pgr* expression. In this context, it is  
435 interesting to note the present observation that the timing of expression of the *mmp15*  
gene was not accelerated by externally adding 17,20 $\beta$ P. This finding appears to support  
the idea that, in addition to Pgr, other regulatory system(s) may be critically involved in  
the *mmp15* gene expression in the ovulating follicles.

A large number of Pgr-regulated, ovulation-related genes induced by LH have been  
440 identified in mammals (Espey and Richards, 2006; Kim et al., 2009; Mishra et al., 2015;  
Robker et al., 2009). Existing evidence also indicates increases in transcript and/or  
protein abundance of various genes at or around the time of ovulation in teleosts (Bobe  
et al., 2006; Garczynski and Goetz, 1997; Nagahama, 1997; Nagahama and Yamashita,  
2008; Takahashi et al., 2013; von Schalburg et al., 2005; Zmora et al., 2007), but to date,  
445 no investigations have been conducted examining whether Pgr might be involved in the  
induced expression of such genes. However, our previous (Hagiwara et al., 2014) and  
present data demonstrate an indispensable role for Pgr in LH-induced expression of the  
*ptger4b* and *mmp15* genes, respectively. A critical role of Pgr as a mediator in the  
process of signal transductions from LH surge to the expression of various  
450 ovulation-related genes in the preovulatory follicles at ovulation remains to be verified  
in other non-vertebrate animals, including amphibians, reptiles, and aves; however, it is  
safe to propose that the role of Pgr in the expression of some, if not all, genes necessary  
for successful ovulation is conserved across vertebrate species from fish to humans.

Substantial evidence has accumulated to indicate the essential role of *Cebpb* in  
455 ovulation in rodents (Sirois and Richards, 1993; Sterneck et al., 1997). *Cebpb* was  
reported to be increased by LH in the preovulatory follicles of the mouse (Fan et al.,  
2009), and targeted disruption of the *Cebpb* gene was shown to cause a failure in  
ovulation (Sterneck et al., 1997). More recently, it has been demonstrated that the  
expression and functional activation of both *Cebpb* and *Cebpa* in the granulosa cells are  
460 essential for LH-, cAMP/PKA-, and ERK1/2-mediated events associated with ovulation  
in the mouse (Fan et al., 2011). Our current study demonstrates that, as in the case of the  
mouse, *Cebpb* expression is LH-inducible in the preovulatory follicles of the fish, and  
exhibits an important role in fish ovulation. At present, there is no information on the  
signaling processes from LH stimulation of the follicles to the *cebpb* gene expression in  
465 the medaka. Precise signaling cascades leading to *Cebpb* expression in the  
LH-stimulated follicles would be worthy of being studied to understand the mechanism  
of vertebrate ovulation from an evolutionary point of view.

In summary, this study explored the mechanisms of *mmp15* mRNA and *Mmp15*  
expression by employing an in vitro follicle ovulation system in medaka. Our data  
470 indicate that *Mmp15* expression is dramatically induced by rLh- and Pgr-dependent  
mechanisms in the preovulatory follicles that are destined to ovulate. This work also  
demonstrates the involvement of the transcription factor *Cebpb* in *Mmp15* expression.  
The present study supports the idea that Pgr plays a critical role in the expression of  
some, if not all, ovulation-related genes, not only in mammalian species but also in fish.

475

### **Acknowledgments**

We thank Dr. Nagahama and Dr. Shibata for their valuable suggestions regarding this study. This work was supported by Grants-in-Aid for Scientific Research 24247010 and 16H04810 from the Ministry of Education, Culture, Sports, Science and Technology of  
480 Japan.

## References

Bobe, J., Montfort, J., Nguyen, T., Fostier, A., 2006. Identification of new participants in the rainbow trout (*Oncorhynchus mykiss*) oocyte maturation and ovulation processes  
485 using cDNA microarrays. *Reprod. Biol. Endocrinol.* 4, 39.  
doi:10.1186/1477-7827-4-39.

Ensembl database. The Wellcome Trust Genome Campus, Hinxton, UK.  
<http://uswest.ensembl.org/index.html>. (accessed 16.06.30).

490

Espey, L.L., Richards, J.S., 2006. Ovulation, in: Neill, J.D. (Eds.), *Physiology of Reproduction*, 3rd ed. Academic Press, Amsterdam, pp. 425–474.

Fan, H.Y., Liu, A., Johnson P.F., Richards, J.S., 2011. CCAAT/enhancer-binding  
495 proteins (C/EBP)- $\alpha$  and  $-\beta$  are essential for ovulation, luteinization, and the expression of key target genes. *Mol. Endocrinol.* 25, 253-268. doi: 10.1210/me.2010-0318.

Fan, H.Y., Liu, Z., Shimada, M., Sterneck, E., Johnson, P.F., Hedrick, S.M., Richards, J.S., 2009. MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female  
500 fertility. *Science* 324, 938-941.

Fujimori, C., Ogiwara, K., Hagiwara, A., Rajapakse, S., Kimura, A., Takahashi, T., 2011. Expression of cyclooxygenase-2 and prostaglandin receptor EP4b mRNA in the ovary of the medaka fish, *Oryzias latipes*: possible involvement in ovulation. Mol. Cell. Endocrinol. 332, 67–77. Doi: 10.1016/j.mce.2010.09.015.

505

Garczynski, M.A., Goetz, F.W., 1997. Molecular characterization of a ribonucleic acid transcript that is highly up-regulated at the time of ovulation in the brook trout (*Salvelinus fontinalis*) ovary. Biol. Reprod. 57, 856–864.

510

Hagiwara, A., Ogiwara, K., Katsu, Y., Takahashi, T., 2014. Luteinizing hormone-induced expression of Ptger4b, a prostaglandin E<sub>2</sub> receptor indispensable for ovulation of the medaka *Oryzias latipes*, is regulated by a genomic mechanism involving nuclear progesterin receptor. Biol. Reprod. 90(6), 126, 1–14. doi: 10.1095/biolreprod.113.115485.

515

Jacobsen, B.M., Horwitz, K.B., 2012. Progesterone receptors, their isoforms and progesterone regulated transcription. Mol. Cell. Endocrinol. 357, 18–29. doi: 10.1016/j.mce.2011.09.016.

520

Kim, J., Bagchi, I.C., Bagchi, M.K., 2009. Control of ovulation in mice by progesterone receptor-regulated gene networks. Mol. Human. Reprod. 15, 821–828. doi: 10.1093/molehr/gap082.

525 Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: how fish eggs are formed. *Gen. Comp. Endocrinol.* 165, 367–389. Doi: 10.1016/j.ygcen.2009.05.022.

Ma, X., Dong, Y., Matzuk, M.M., Kumar, T.R., 2004. Targeted disruption of luteinizing hormone hormone beta-subunit leads to hypogonadism, defects in gonadal  
530 steroidogenesis, and infertility. *Proc Natl Acad Sci US A.* 101, 17294-17299.

Mishra, B., Park, Y., Wilson, K., Jo, M., 2015. X-linked lymphocyte regulated gene 5c-like (Xlr5c-like) is a novel target of progesterone action in granulosa cells of periovulatory rat ovaries. *Mol. Cell. Endocrinol.* 412, 226–238.  
535 doi:10.1016/j.mce.2015.05.008

Nagahama, Y., 1997.  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action. *Steroids* 62, 190–196.

540 Nagahama, Y., Yamashita, M., 2008. Regulation of oocyte maturation in fish. *Dev Growth Differ.* 50, S195-S219. doi: 10.1111/j.1440-169X.2008.01019.x

Ogiwara, K., Fujimori, C., Rajapakse, S., Takahashi, T., 2013. Characterization of luteinizing hormone and luteinizing hormone receptor and their indispensable role in the  
545 ovulatory process of the medaka. *PLoS ONE* 8(1), e54482.  
doi:10.1374/journal.pone.0054482.

Ogiwara, K., Hagiwara, A., Rajapakse, S., Takahashi, T., 2015. The role of urokinase



plasminogen activator and plasminogen activator inhibitor-1 in follicle rupture during  
550 ovulation in the teleost medaka. *Biol. Reprod.* 92(1), 10, 1–17. doi:  
10.1095/biolreprod.114.121442.

Ogiwara, K., Minagawa, K., Takano, N., Kageyama, T., Takahashi, T., 2012. Apparent  
involvement of plasmin in early-stage of follicle rupture during ovulation in medaka.  
555 *Biol. Reprod.* 86(4), 113, 1–10. doi: 10.1095/biolreprod.111.093880.

Ogiwara, K., Takahashi, T., 2007. Specificity of the medaka enteropeptidase serine  
protease and its usefulness as a biotechnology tool for fusion-protein cleavage. *Proc.*  
*Natl. Acad. Sci. USA* 104, 7021–7026. doi: 10.1073/pnas.0610447104

560

Ogiwara, K., Takano, N., Shinohara, M., Murakami, M., Takahashi, T., 2005.  
Gelatinase A and membrane-type matrix metalloproteinases 1 and 2 are responsible for  
follicle rupture during ovulation in the medaka. *Proc. Natl. Acad. Sci. USA* 102,  
8442–8447. doi:10.1073/pnas.0502423102

565

Potts, G.O., Creange, J.E., Hardomg, H.R., Schane, H.P., 1978. Trilostane, an orally  
active inhibitor of steroid biosynthesis. *Steroids* 32, 257–267.

Richards, JS., Hormonal control of gene expression in the ovary. 1994. *Endocr. Rev.* 15,  
570 725-751.

Robker, R.L., Akison, L.K., Russell, D.L., 2009. Control of oocyte release by  
24

progesterone receptor-regulated gene expression. *Nucl. Recept. Signal.* 7, e012. doi: 10.1621/nrs.07012.

575

Sakai, N., Iwamatsu, T., Yamauchi, K., Nagahama, Y., 1987. Development of the steroidogenic capacity of medaka (*Oryzias latipes*) ovarian follicles during vitellogenesis and oocyte maturation. *Gen. Comp. Endocrinol.* 66, 333–342.

580 Sirois, J., Richards, J.S., 1993. Transcriptional regulation of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. Evidence for a role of a cis acting C/EBP $\beta$  promoter element. *J. Biol. Chem.* 268, 21931-21938.

585 Sterneck, E., Tassarollo, L., Johnson, P.F., 1997. An essential role for C/EBP $\beta$  in female reproduction. *Genes Dev.* 11, 2153-2162.

Takahashi, A., Kanda, S., Abe, T., Oka, Y., 2016. Evolution of the hypothalamic-pituitary-gonadal axis regulation in vertebrates revealed by knockout medaka. *Endocrinol.* 157, 3994-4002. doi:10.1210/en.2016-1356.

590

Takahashi, T., Fujimori, C., Hagiwara, A., Ogiwara, K., 2013. Recent advances in the understanding of teleost medaka ovulation: the role of proteases and prostaglandins. *Zool. Sci.* 30, 239–247. doi: 10.2108/zsj.30.239.

595 TFBIND [Internet]. Tsunoda T: Medical Science Mathematics, RIKEN Center. <http://tfbind.hgc.jp/> (accessed 16.06.30)

von Schalburg, K.R., Rise, M.L., Brown, G.D., Davidson, W.S., Koop, B.F., 2005. A  
comprehensive survey of the genes involved in maturation and development of the  
600 rainbow trout ovary. *Biol. Reprod.* 72, 687–699. doi: 10.1095/biolreprod.104.034967.

Zhang, Z., Zhu, B., Ge, W., 2015. Genetic analysis of zebrafish gonadotropin (FSH and  
LH) functions by TALEN-mediated gene disruption. *Mol. Endocrinol.* 29, 76-98. doi:  
10.1210/me.2014-1256.

605

Zmora, N., Kazeto, Y., Kumar, R.S., Schulz, R.W., Trant, J.M., 2007. Production of  
recombinant channel catfish (*Ictalurus punctatus*) FSH and LH in S2 Drosophila cell  
line and an indication of their different actions. *J. Endocrinol.* 194, 407–416.  
doi:10.1677/JOE-07-0171.

610

615

## Figures

Fig. 1. An outline of the preovulatory follicle culture experiments carried out in this  
study. The follicles isolated from ovaries 22 h before ovulation were incubated in the  
620 presence of medaka recombinant LH (rLh) (100 µg/ml) with or without chemicals. The

expected timing of the LH surge, GVBD, and ovulation in vivo and in vitro are shown. Red arrows indicate the follicle layers of ovulated follicles. Arrowheads indicate the time points of analyses.

625 Fig. 2. Inhibitory effect of TSL on the induction of *mmp15*/Mmp15 expression and  
ovulation in the preovulatory follicles treated with rLh. (A) Preovulatory follicles  
isolated from medaka ovaries at 22 h before ovulation were incubated in the presence of  
rLh (100 µg/ml) with TLS (25 µM) or TLS (25 µM) and 17, 20βP (1 µM). After 30 h of  
incubation, total RNA was extracted from the follicles and was used for real-time  
630 RT-PCR analysis of *mmp15* mRNA. The expression levels were normalized to those of  
*actb* and were expressed as the fold-change compared with the levels of the 0 h follicles.  
Asterisks indicate a significant difference at  $P < 0.01$  (\*\*) or  $P < 0.05$  (\*) compared with  
the expression of the 0 h follicles (ANOVA and Dunnett's post hoc test, N=6). (B)  
Preovulatory follicles isolated from ovaries at 22 h before ovulation were cultured in the  
635 same conditions as (A). After 30 h of incubation, the extract of the follicle layers was  
prepared for western blot analysis of the Mmp15 protein. Actb protein was also detected  
as a control. Three separate experiments were performed to confirm the reproducibility  
of the findings and the result of one experiment is shown. The positions of various  
molecular masses (M) are indicated. The dotted line represents cropping of a single gel.  
640 (C) Preovulatory follicles isolated from ovaries at 22 h before ovulation were incubated  
in the same condition as (A). After 30 h of incubation, ovulation rates were determined.  
Asterisks indicate a significant difference at  $P < 0.01$  (\*\*) compared with the follicles  
incubated with any additives (ANOVA and Dunnett's post hoc test, N=6).

645 Fig. 3. Inhibitory effect of RU-486 on the induction for *mmp15*/Mmp15 expression in  
the preovulatory follicles treated with rLh. (A) Preovulatory follicles isolated from  
medaka ovaries at 22 h before ovulation were incubated in the presence of rLh (100  
 $\mu\text{g/ml}$ ) with RU486 (100  $\mu\text{M}$ ) or RU-486 (100  $\mu\text{M}$ ) and 17, 20 $\beta\text{P}$  (1  $\mu\text{M}$ ). After 30 h of  
incubation, total RNA was extracted from the follicles and used for the real-time  
650 RT-PCR analysis of *mmp15* mRNA. The expression levels were normalized and  
expressed as the fold-change compared with the levels of the 0 h follicles. Asterisks  
indicate a significant difference at  $P < 0.05$  (\*) (t-test,  $N=6$ ). (B) Preovulatory follicles  
isolated from ovaries at 22 h before ovulation were cultured in the same conditions as  
(A). After 30 h of incubation, extracts of the follicle layers were prepared for western  
655 blot analysis of Mmp15 protein. Actb protein was detected as a control. At least three  
separate experiments were performed to confirm the reproducibility of the findings and  
the result of one experiment is shown. The positions of various molecular masses (M)  
are indicated. The dotted line represents cropping of a single gel.

660 Fig. 4. Inhibitory effect of RU-486 on the induction for *mmp15*/Mmp15 expression in  
the preovulatory follicles that had been exposed to LH in vivo. (A) Preovulatory  
follicles isolated from medaka ovaries at 12 h before ovulation were incubated with or  
without RU-486 (100  $\mu\text{M}$ ). After 18 h of incubation, total RNA was extracted from the  
follicles and used for the real-time RT-PCR analysis of *mmp15* mRNA. The expression  
665 levels were normalized and expressed as the fold-change compared with the levels of  
the follicles immediately after isolation from the ovary at 12 h before ovulation.  
Asterisks indicate a significant difference at  $P < 0.01$  (\*\*) (t-test,  $N=6$ ). (B) Preovulatory  
follicles isolated from ovaries at 12 h before ovulation were cultured in the same

conditions as (A). After 18 h of incubation, extracts of the follicle layers were prepared  
670 for western blot analysis of Mmp15 protein. Actb protein was detected as a control. At  
least three separate experiments were performed to confirm the reproducibility of the  
findings and the result of one experiment is shown. The positions of various molecular  
masses (M) are indicated.

675 Fig. 5. Inhibitory effect of RU-486 on the induction of *pgr*/Pgr expression in  
preovulatory follicles treated with rLh. (A) Extracts of preovulatory follicles isolated  
from ovaries at the indicated time points were immunoprecipitated with rat anti-medaka  
Pgr antibody coupled with Protein G-Sepharose, and the precipitated materials were  
analyzed by western blotting using mouse anti-medaka Pgr antibody (upper panel). Actb  
680 protein was detected as a control (lower panel). At least three separate experiments were  
performed to confirm the reproducibility of the findings and the result of one  
experiment is shown. The positions of various molecular masses (M) are indicated. (B)  
Preovulatory follicles were isolated from ovaries at 22 h before ovulation and incubated  
with rLh (100 µg/ml). The follicles were collected every 3 h to prepare tissue extracts.  
685 Immunoprecipitation followed by western blot analysis was conducted as in (A) (upper  
panel). Actb protein was detected as a control (lower panel). At least three separate  
experiments were performed to confirm the reproducibility of the findings and the result  
of one experiment is shown. The positions of various molecular masses (M) are  
indicated. The dotted line represents cropping of a single gel. (C) Preovulatory follicles  
690 isolated from ovaries at 22 h before ovulation were incubated with rLh (100 µg/ml) with  
or without RU-486 (100 µM). After 12 h of incubation, total RNA was extracted from  
the follicles and used for real-time RT-PCR analysis of *pgr* mRNA. The expression

levels were normalized and expressed as the fold-change compared with the levels of the 0 h follicles (N=6). (D) Preovulatory follicles isolated from ovaries at 22 h before ovulation were cultured in the same conditions as in (C). After 12 h of incubation, extracts of the follicles were immunoprecipitated and western blot analyses were conducted as in (A). Actb protein was detected as a control (lower panel). At least three separate experiments were performed to confirm the reproducibility of the findings and the result of one experiment is shown. The positions of various molecular masses (M) are indicated. The dotted line represents cropping of a single gel.

Fig. 6. Binding of Pgr to the promoter region of the *mmp15* gene.

(A) The diagram illustrates the positions of seven ChIP primer pairs (P-1 to P-7) in the 1.5 kb upstream region of the transcription start site (indicated as +1) of the *mmp15* gene. Thick vertical lines numbered from 1 to 8 represent the positions of putative progesterone response elements (PREs). Thin vertical lines indicate the positions of putative Cebp $\beta$  binding site. (B) Preovulatory follicles isolated at 4 h before ovulation were used for the ChIP assays using the primer pairs 1 to 7. The sheared DNA that was immunoprecipitated with mouse anti-medaka Pgr antibody was analyzed by real-time PCR. As a negative control, the assay was also performed using primer pair 8 (P-8). Asterisks indicate a significant difference at  $P < 0.01$  (\*\*), compared with the enrichments of the negative control (ANOVA and Dunnett's post hoc test, N=6). (C) Preovulatory follicles isolated at 23, 15, 10, or 4 h before ovulation were used for the ChIP assays using the primer pair 1. The sheared DNA that was immunoprecipitated with the mouse anti-medaka Pgr antibody was analyzed by real-time PCR. Asterisks indicate a significant difference at  $P < 0.01$  (\*\*), compared with the enrichments of the -23, -15,

and -10 h follicles (ANOVA and Tukey's post hoc test, N=6). (D) OLHNI-2 cells stably expressing medaka Pgr was cultured with or without 17, 20βP. After culture, the expression level for *mmp15* was examined by real-time RT-PCR. Asterisks indicate a significant difference at P<0.05 (\*) (t-test, N=4).

Fig. 7. Expression of *cebpb* and Cebpb in the preovulatory follicles of spawning medaka.

Real-time RT-PCR analysis of *cebpb* mRNA was conducted using total RNAs extracted from preovulatory follicles isolated from spawning medaka ovaries at the indicated time points. The expression levels were normalized and expressed as the fold-change compared with the levels of the -23 h follicles (set as 1). Asterisks indicate a significant difference at P<0.01 (\*\*) or P<0.05 (\*) compared with the levels of the -23 h follicles (ANOVA and Dunnett's post hoc test, N=4). (B) Preovulatory follicles were isolated from spawning medaka ovaries 22 h before ovulation and were incubated with or without medaka rLh (100 μg/mL). Total RNA was extracted from the in vitro incubated follicles taken every 3 h and used for real-time RT-PCR of *cebpb* mRNA. The expression levels were normalized to that of *actb* and expressed as the fold change compared to the levels of the 0 h follicles. Asterisks indicate a significant difference at P<0.01 (\*\*) compared with the levels of the -22 h follicles (ANOVA and Dunnett's post hoc test, N=5). (C) Recombinant Cebpb (rCebpb) was subjected to SDS-PAGE, followed by Coomassie Brilliant blue (CBB) staining (left panel) or Western blot analysis (WB) using either an anti-Cebpb antibody (Cebpb-AB) or the antibody that was previously incubated with the antigen (Cebpb-AB(abs)) (right panel). Three separate experiments were performed to confirm the reproducibility of the findings, and



the result of one experiment is shown. The positions of various molecular masses (M) are also shown. The dotted line represents the cropping of a single gel. (D) Extracts of the follicle layers of preovulatory follicles collected at the various time points indicated were subjected to Western blot analysis using anti-medaka Cebpb antibody (upper  
745 panel) or anti-Actb antibody (lower panel). At least three separate experiments were performed to confirm the reproducibility of the findings and the result of one experiment is shown. The positions of the various marker proteins (M) are shown. The dotted line represents the cropping of a single gel.

750 Fig. 8. Binding of Cebpb to the promoter region of the *mmp15* gene.

(A) Preovulatory follicles isolated at 4 h before ovulation were used for the ChIP assays using the primer pairs 1–7. Positions of the primers are illustrated in Fig. 6A. The sheared DNA that was immunoprecipitated with mouse anti-medaka Cebpb antibody was analyzed by real-time PCR. As a negative control, the assay was performed using  
755 primer pair 8. Asterisks indicate a significant difference at  $P < 0.01$  (\*\*) or  $P < 0.05$  (\*) compared with the enrichments of the negative control (ANOVA and Dunnett's post hoc test,  $N=6$ ).

(B) Preovulatory follicles isolated 10 h or 4 h before ovulation were used for the ChIP assay using the primer pairs 1, 7, and 8. The positions of the primers are illustrated in Fig. 6A. The sheared DNA that was immunoprecipitated with the  
760 anti-Cebpb antibody was analyzed by real-time PCR. Asterisks indicate a significant difference at  $P < 0.01$  (\*\*) or  $P < 0.05$  (\*) compared with the respective control (t-test,  $N=4$ ).

(C) HEK293T cells were transiently co-transfected with pCMV-Pgr, pGL3-MT2, pRL mock, and either pCMV tag4 or pCMV-Cebpb. After 24 h incubation, the cells were further treated with or without 17,20 $\beta$ P (1  $\mu$ M) for 24 h. Luciferase activity was

765 measured using the extracts of the treated cells. Relative enzyme activities are shown as compared with those of the extract obtained from the Pgr-expressing 293T cells incubated without 17, 20 $\beta$ P. Asterisks indicate a significant difference at P<0.05 (\*) or P<0.01 (\*\*) compared with the pCMV-Pgr-transfected follicles incubated without 17,20 $\beta$ P (ANOVA and Dunnett's post hoc test, N=4).

770

**Supplemental Table S1.** Primers used in this study.

Primer name	gene	Sequence	Accession No.
<u>5'-RACE</u>			
Mmp15-GSP-1	<i>mmp15</i>	5'-TCTGACACTTTAATCGCC-3'	AB072928
Mmp15-GSP-2	<i>mmp15</i>	5'-GCTTGTGTTGTCTTTGCGCGACTG-3'	AB072928
Mmp15-GSP-3	<i>mmp15</i>	5'-GCAAATACAGCCTCATTGCGTT-3'	AB072928
<u>Real-time PCR</u>			
Mmp15 real-t SS	<i>mmp15</i>	5'-ACGGCTCCCTCCAGTTTTAC-3'	AB072928
Mmp15 real-t AS	<i>mmp15</i>	5'-GGTGTGTTTCTGCGTCTTC-3'	AB072928
Cebpb real-t SS	<i>cebpb</i>	5'-TCAGCAACTGGACAATGTTA-3'	XM_011477517
Cebpb real-t AS	<i>cebpb</i>	5'-CAGTGTCATTGTAAACTGG-3'	XM_011477517
$\beta$ -actin real-t SS	<i>actb</i>	5'-TGACGGAGCGTGGCTACTC-3'	D89627
$\beta$ -actin real-t AS	<i>actb</i>	5'-TCCTTGATGTCACGGACAATTT-3'	D89627
<u>ChIP</u>			
Primer pair-1 SS for Mmp15	<i>mmp15</i>	5'-GAAAGTCATGACGTCCTGG-3'	
Primer pair-1 AS for Mmp15	<i>mmp15</i>	5'-GGCTCCGCTCCTCCAGAGCC-3'	
Primer pair-2 SS for Mmp15	<i>mmp15</i>	5'-GTTAGGTTAGGCACACTTAA-3'	
Primer pair-2 AS for Mmp15	<i>mmp15</i>	5'-TGGAATGTAAACCATTACAA-3'	
Primer pair-3 SS for Mmp15	<i>mmp15</i>	5'-CATGTTATAACACATGGATG-3'	
Primer pair-3 AS for Mmp15	<i>mmp15</i>	5'-AAGATTGACTAGCAAGAAGG-3'	
Primer pair-4 SS for Mmp15	<i>mmp15</i>	5'-CCAATGCTAATGGATACGCT-3'	
Primer pair-4 AS for Mmp15	<i>mmp15</i>	5'-ATGTGAACCACTTTGATGAT-3'	
Primer pair-5 SS for Mmp15	<i>mmp15</i>	5'-CTAAAGTTTCCCATTAACCT-3'	
Primer pair-5 AS for Mmp15	<i>mmp15</i>	5'-GTCATATTTCACTCCCACAA-3'	
Primer pair-6 AS for Mmp15	<i>mmp15</i>	5'-CTTTTGTACCCGTCTCCAT-3'	
Primer pair-6 AS for Mmp15	<i>mmp15</i>	5'-GACAAGATGGCCACCAGCTT-3'	
Primer pair-7 AS for Mmp15	<i>mmp15</i>	5'-TTTCGTGCGGATGATATATC-3'	
Primer pair-7 AS for Mmp15	<i>mmp15</i>	5'-TTGCAGCACATTAAGATTGG-3'	
Primer pair-8 SS for Mmp15	<i>mmp15</i>	5'-AGATATTGCAGTTGCTATACGG-3'	
Primer pair-8 AS for Mm-15	<i>mmp15</i>	5'-TCATCTGTGTTTCTTTTCAC-3'	
<u>Recombinant protein preparation</u>			
Cebpb First M SS	<i>cebpb</i>	5'-ATGGAAGTGGCCGGCTTGTA-3'	XM_011477517
Cebpb stop AS	<i>cebpb</i>	5'-CTAACATTGTCCAGTTGCTG-3'	XM_011477517
<u>Luciferase assay</u>			

---

Mmp15-Luc SS	<i>mmp 15</i>	5'-GTGGGAGTGAAATATGACTA-3'
Mmp15-Luc AS	<i>mmp 15</i>	5'-GGCTCCGCTCCTCCAGAGC-3'

---

Fig. 1

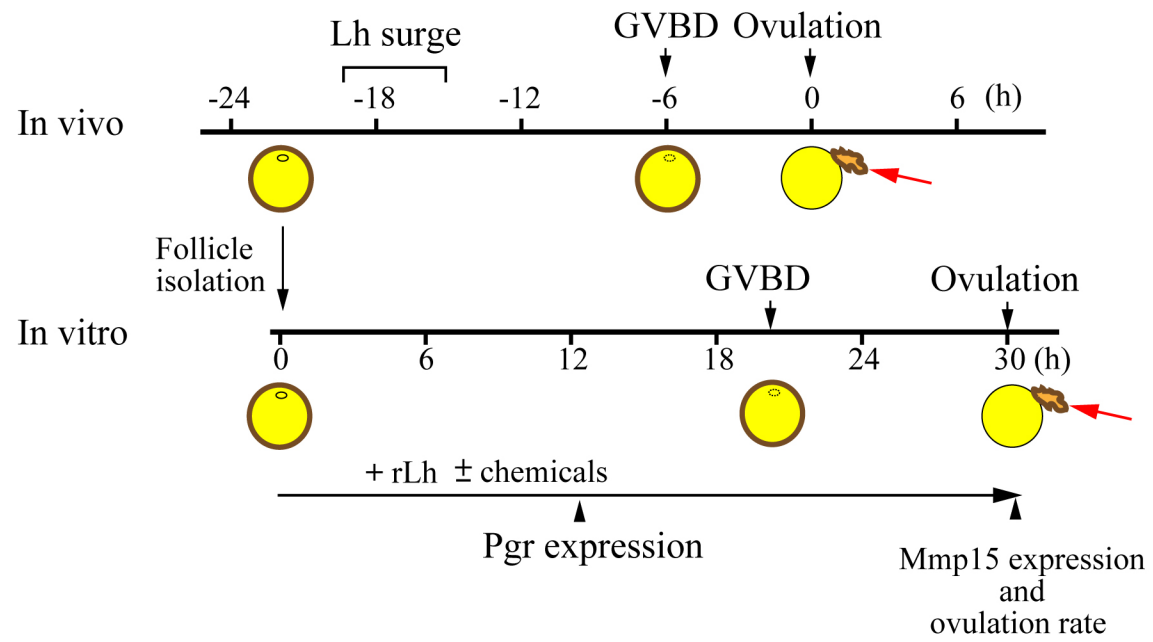


Fig. 2

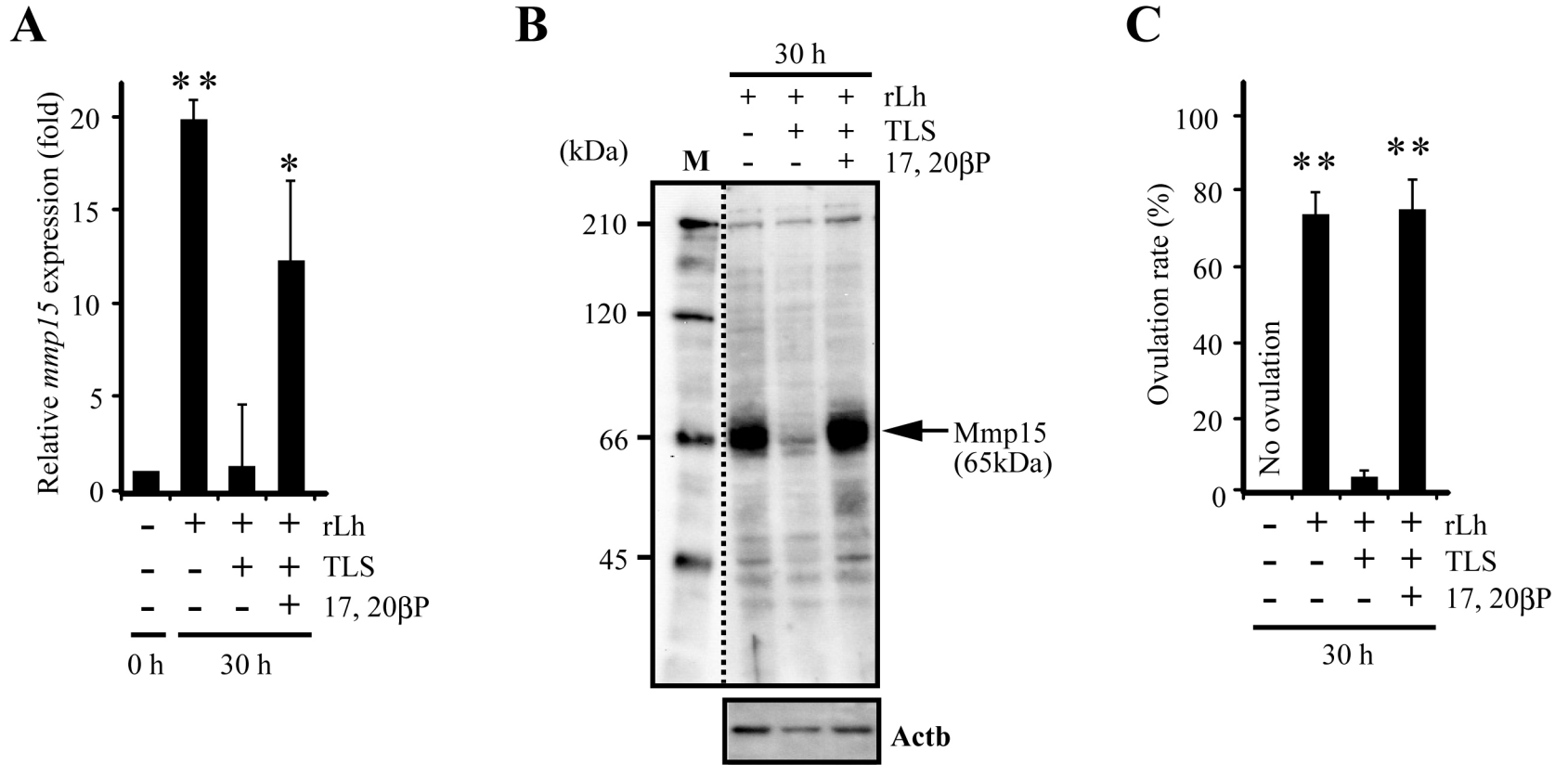
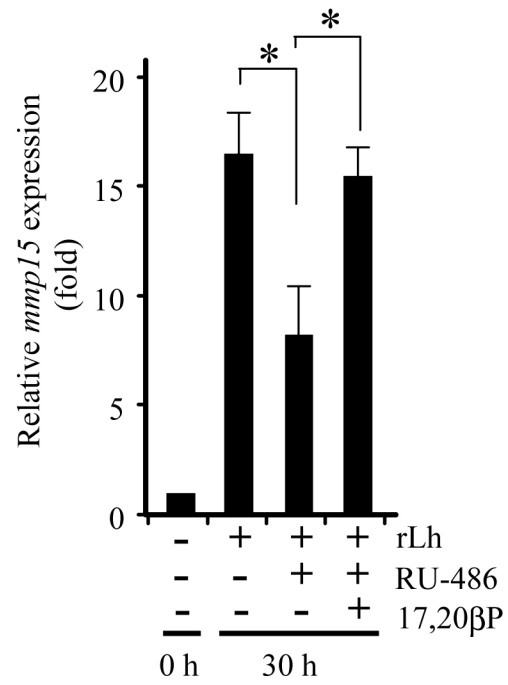


Fig. 3

A



B

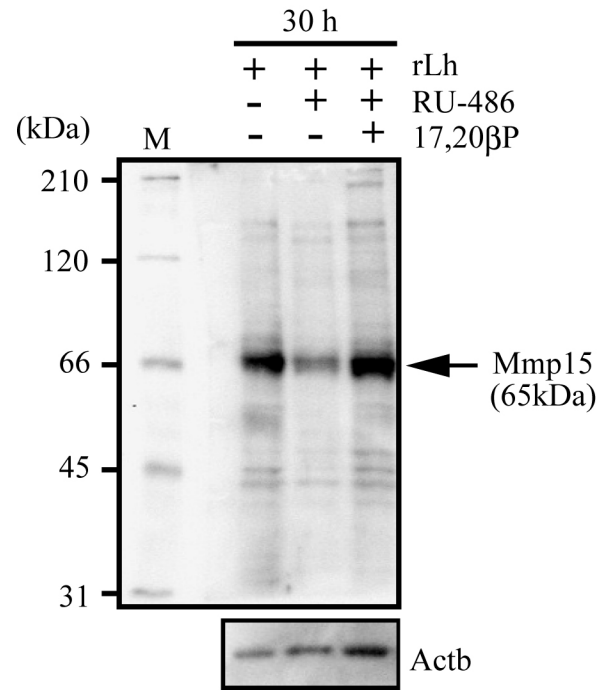


Fig. 4

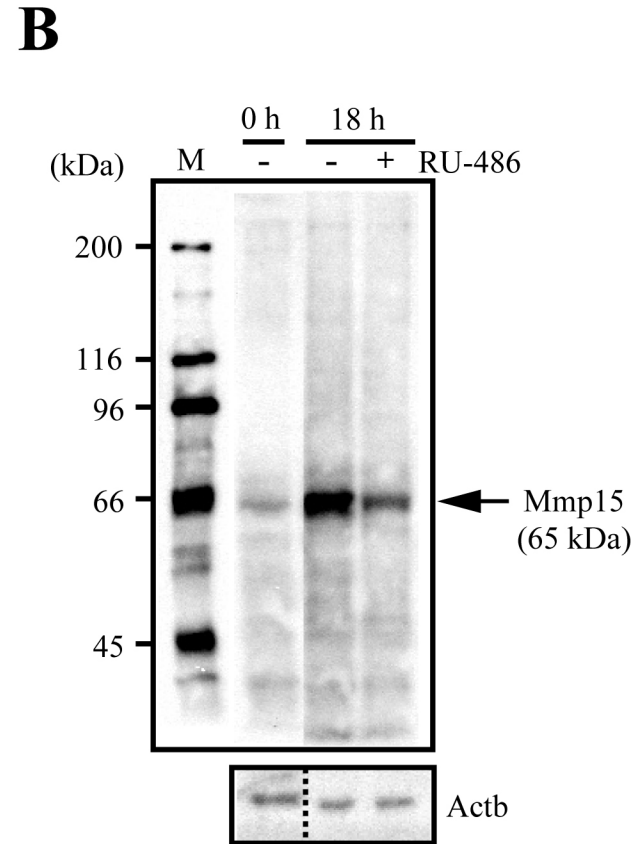
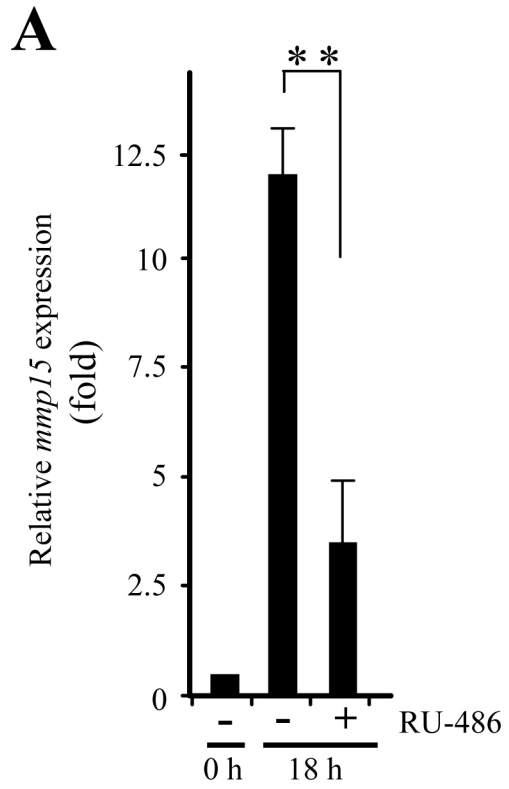




Fig. 5

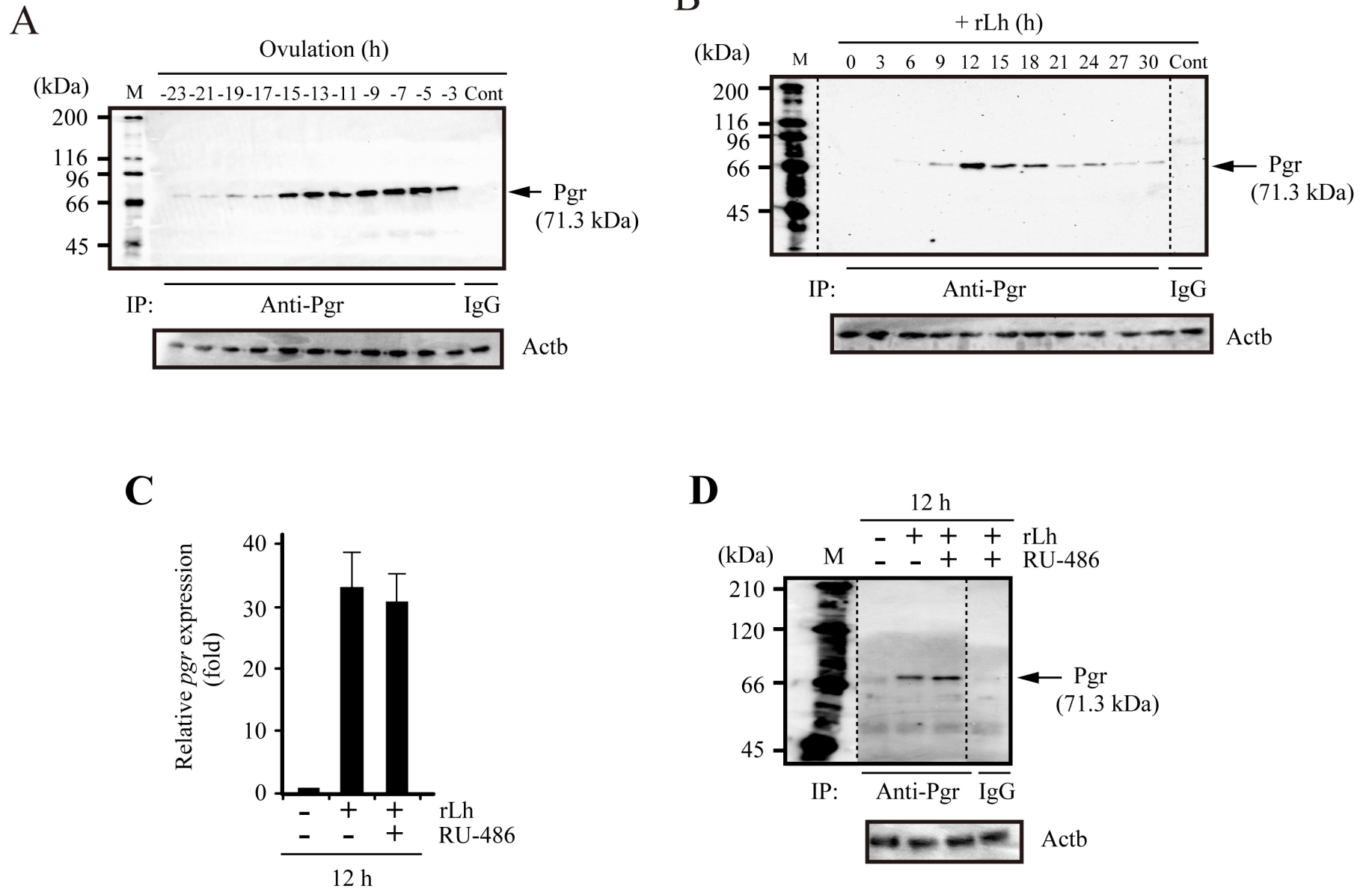
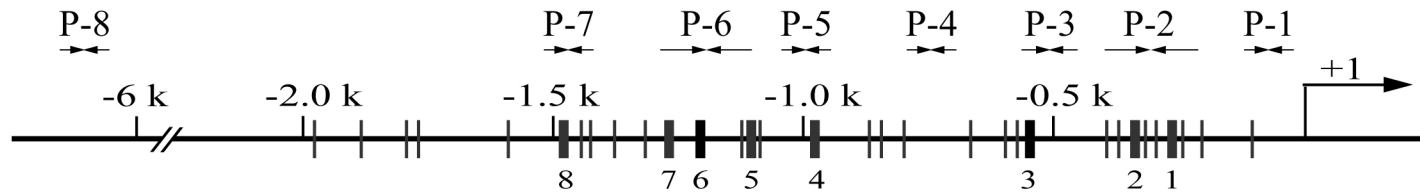
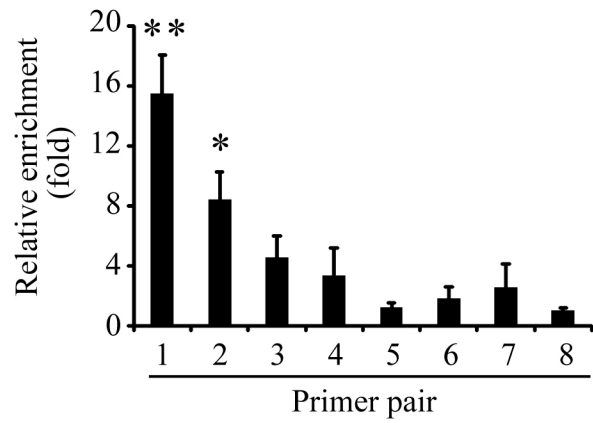


Fig. 6

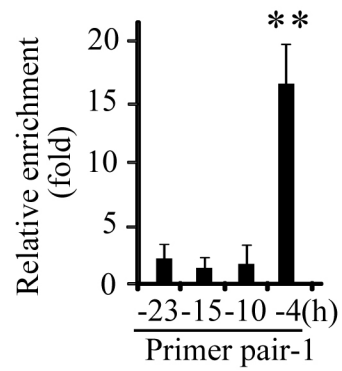
A



B



C



D

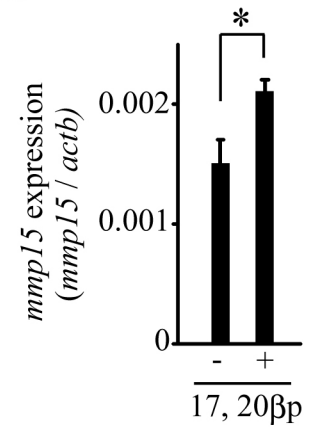


Fig. 7

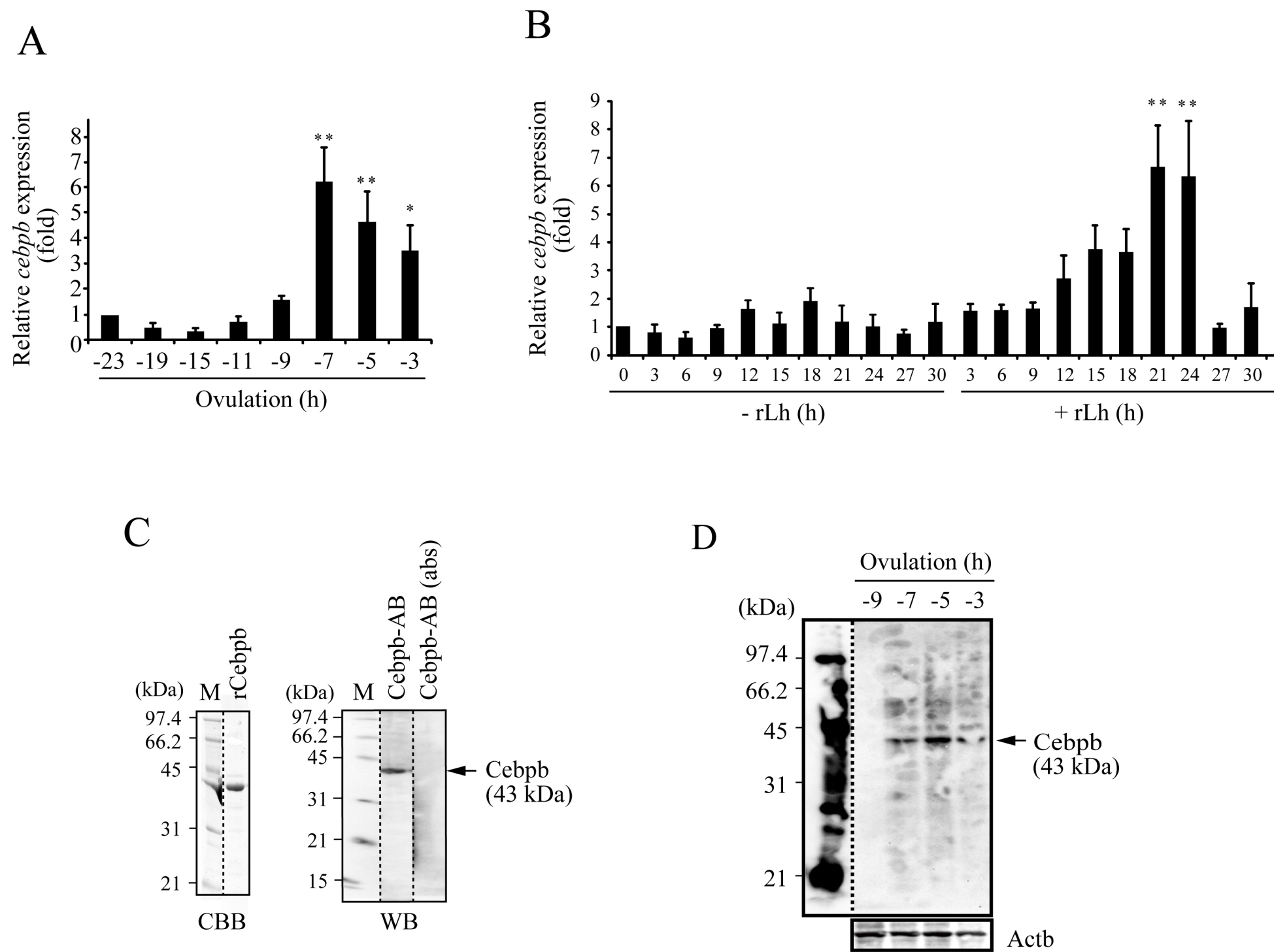
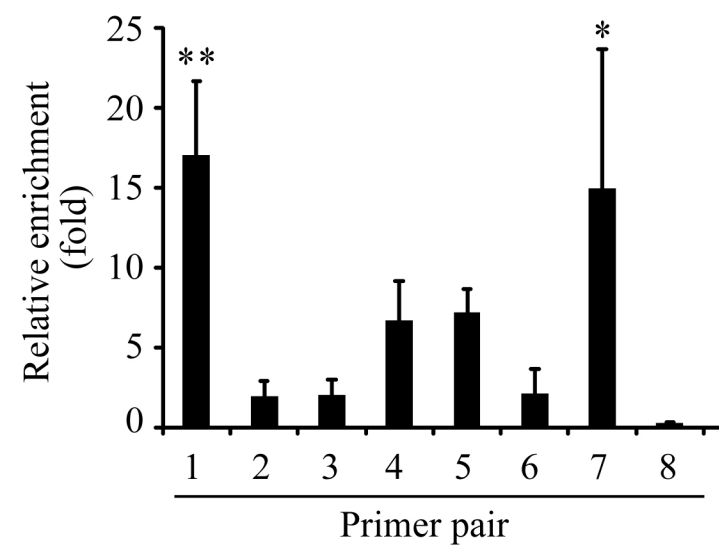
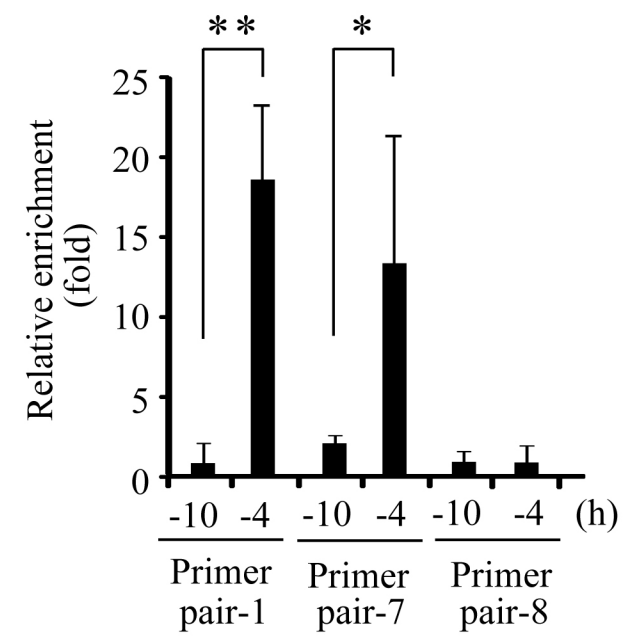


Fig. 8

A



B



C

