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Possible involvement of insulin-like growth factor 2 mRNA-binding protein 3 in zebrafish oocyte maturation as a novel *cyclin B1* mRNA-binding protein that represses the translation in immature oocytes

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ABSTRACT

In immature zebrafish oocytes, dormant *cyclin B1* mRNAs localize to the animal polar cytoplasm as aggregates. After hormonal stimulation, *cyclin B1* mRNAs are dispersed and translationally activated, which are necessary and sufficient for the induction of zebrafish oocyte maturation. Besides cytoplasmic polyadenylation element-binding protein (CPEB) and *cis*-acting elements in the 3' untranslated region (UTR), Pumilio1 and a *cis*-acting element in the coding region of *cyclin B1* mRNA are important for the subcellular localization and timing of translational activation of the mRNA. However, mechanisms underlying the spatio-temporal control of *cyclin B1* mRNA translation during oocyte maturation are not fully understood. We report that insulin-like growth factor 2 mRNA-binding protein 3 (IMP3), which was initially described as a protein bound to *Vg1* mRNA localized to the vegetal pole of *Xenopus* oocytes, binds to the 3' UTR of *cyclin B1* mRNA that localizes to the animal pole of zebrafish oocytes. IMP3 and *cyclin B1* mRNA co-localize to the animal polar cytoplasm of immature oocytes, but in mature oocytes, IMP3 dissociates from the mRNA despite the fact that its protein content and phosphorylation state are unchanged during oocyte maturation. IMP3 interacts with Pumilio1 and CPEB in an mRNA-dependent manner in immature oocytes but not in mature oocytes. Overexpression of IMP3 and injection of anti-IMP3 antibody delayed the progression of oocyte maturation. On the basis of these results, we propose that IMP3 represses the translation of *cyclin B1* mRNA in immature zebrafish oocytes and that its release from the mRNA triggers the translational activation.

Key words: CPEB; IMP3; Pumilio; MPF; mRNA localization; translational control

1. **Introduction**

Immature oocytes become mature (fertilizable) by the activity of maturation-promoting factor (MPF), a protein kinase consisting of the catalytic subunit Cdc2 and regulatory subunit Cyclin B1. Since Cyclin B1 is absent in immature oocytes of fish and amphibians except *Xenopus laevis*, its *de novo* synthesis is essential for the initiation of oocyte maturation in these species [1,2,3]. Protein synthesis during oocyte maturation chiefly depends on the translational activation of maternal mRNAs, which are synthesized during oocyte growth and stored in dormant forms in the oocytes until activated at timings specific to each mRNA. The translational activation of maternal mRNAs, including *cyclin B1* mRNA, is triggered by cytoplasmic polyadenylation, which is mainly regulated by the cytoplasmic polyadenylation element-binding protein (CPEB) that resides in the 3' untranslated region (UTR) [4]. Besides CPEB, however, other RNA-binding proteins specific to each mRNA are required for the accurate timings of translation. For example, the strict temporal order of *mos*, *cyclin B1* and *wee1* mRNA translation, which is important to ensure the normal progression of oocyte maturation, is regulated by CPEB in cooperation with certain partners, Musashi for *mos* [5], Pumilio1 for *cyclin B1* [6] and Zar2 for *wee1* [7].

Fish oocytes are characterized by the micropyle, a sperm entry hole on the egg chorion at the animal pole (Supplementary Figure 1A), while amphibian (*Xenopus*) oocytes lack it. In zebrafish, dormant *cyclin B1* mRNAs aggregate and localize to the animal polar cytoplasm beneath the micropyle (Supplementary Figure 1B), whereas similar aggregation and localization have not been reported for *Xenopus cyclin B1* mRNA. After hormonal stimulation, *cyclin B1* mRNAs disperse, leading to translational activation of the mRNA, an event necessary and sufficient for the induction of oocyte maturation in zebrafish [8]. We have previously shown that Pumilio1 bound to the 3' UTR and an unidentified protein(s) bound to the coding region of *cyclin B1* mRNA are required for the correct localization and temporal regulation of translational activation of the mRNA [9,10,11]; however, we are still far from a comprehensive understanding of mechanisms that ensure the spatio-temporally regulated translational activation of *cyclin B1* mRNA during

zebrafish oocyte maturation. Here, we report that IMP3 is a novel *cyclin B1* mRNA-binding protein in immature zebrafish oocytes and suggest that it represses the translation of *cyclin B1* mRNA in immature oocytes.

2. Materials and methods

2.1. Oocyte culture and extraction

All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University (No. 13-0099). Full-grown oocytes were manually isolated from ovaries and placed in zebrafish Ringer's solution [8]. Maturation was induced by incubating the oocytes in culture medium (90% Leibovitz's L-15, 0.5% bovine serum albumin (BSA), 100 μg/ml gentamycin; pH 9.0) containing 1 μg/ml 17α,20β-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish. The progression of maturation was assessed by the occurrence of germinal vesicle breakdown (GVBD). At appropriate times after hormonal stimulation, oocytes were homogenized with a pestle to obtain oocyte extracts (1 μl/oocyte), as described previously [9].

2.2. Production of recombinant proteins and antibodies

To produce a full-length IMP3 with a Flag tag at the N terminus (Flag-IMP3), an ORF of zebrafish IMP3 (NM_131491.2) was amplified with a primer set which introduces *Cla*I and *Xho*I sites (5'-ATCGATatgaataagctgtacatcgggaa-3' and 5'-CTCGAGtttcctcctggcgactg-3'). The PCR product was cloned into pGEM-T easy vector (Promega), digested with *Cla*I and *Xho*I, and ligated into pCS2-Flag-N.

To produce a truncated IMP3 tagged with a glutathione-S-transferase (GST) at the N terminus (GST-IMP3) or a polyhistidine (His) at the C terminus (IMP3-His), a cDNA fragment of 589-1227 of zebrafish IMP3 was amplified with a primer set (5'-caccctacgcttgctggtaccg-3' and 5'-ttccgactccatctgcg-3'). The PCR product was cloned into pENTR/D-TOPO vector (Invitrogen) and the resulting plasmid was

recombined with the destination vectors pDEST15 for GST-IMP3 and pET161-DEST for IMP3-His, using the Gateway cloning system (Invitrogen).

GST-IMP3 and IMP3-His expressed in *Escherichia coli* BL21 (DE3) were purified by SDS-PAGE followed by electro-elution in Tris-glycine buffer without SDS, and they were injected into mice to produce polyclonal antibodies. IMP3-His was electroblotted onto an Immobilon membrane and used to affinity purify the antibodies.

Using a full-length cDNA clone (AB044534) in pBluescript SK, an ORF of goldfish CPEB was amplified with a primer that introduces a *Bgl*II site just before the first ATG codon (5'-gaAGATCTatggcgttttctctgagc-3') and the T7 primer. The resulting cDNA was digested with *Bgl*II and *Eco*RV and ligated into *Bam*HI-*Eco*RV-cut pET3a vector (Novagen). Monoclonal antibodies were raised against *E. coli*-produced proteins. Two monoclonal antibodies (GFCPEB 3D3 and 9G3) recognize zebrafish CPEB (Supplementary Figure 2A).

Digoxigenin (DIG) was coupled with BSA or keyhole limpet hemocyanin using DIG-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Roche) and was injected into guinea pigs and rabbits to produce anti-DIG antibodies. Since an antiserum from the guinea pig injected with DIG-conjugated BSA showed high specificity to DIG, it was used in combination with Alexa 488-labeled secondary antibody to detect DIG-labeled *cyclin B1* probes under a fluorescent microscope for section *in situ* hybridization analysis, as described below.

2.3. Immunoblotting and immunoprecipitation

Immunoblotting was performed as described previously [12], using anti-IMP3, anti-Pumilio1 (Pum2A5) [13], anti-CPEB (GFCPEB 3D3 or 9G3), anti-Cdc2 (MC2-21) [1], anti-phospho-MAP kinase (#9101; Cell Signaling Technology), anti-γ-tubulin (T6557; Sigma-Aldrich) or anti-Flag (F1804; Sigma-Aldrich) antibodies. The interaction of IMP3 with Pumilio1 or CPEB was examined by immunoprecipitation, as

described previously [12]. To detect phosphorylation of IMP3, immature and mature oocyte extracts were immunoprecipitated with anti-IMP3 antibody. The resulting precipitates were treated with λ-phosphatase (200 units, New England Biolabs) for 1 h at 30°C, separated by SDS-polyacrylamide gel containing 20 μM Phos-tag acrylamide (NARD institute) and 40 μM $MnCl_2$, and immunoblotted with anti-IMP3 antibody.

2.4. Sucrose density gradient ultracentrifugation

Zebrafish ovaries were homogenized in 500 μl of gradient buffer (80 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, 20mM EDTA; pH 7.4) and centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was loaded onto 4 ml of 0.1–1.9 M sucrose density gradient prepared in gradient buffer. After centrifugation at 35,000 rpm in a Hitachi S52ST rotor for 5 h at 4°C, 450-μl fractions were collected from the bottom of the tube. *cyclin B1* mRNA contents in each fraction were analyzed by northern blotting, according to the procedure reported previously [8]. The fractions were also analyzed by immunoblotting to detect Cdc2, Pumilio1, CPEB and IMP3 and by SDS-PAGE followed by silver staining to detect total protein.

2.5. UV cross-linking assay

mRNAs encoding Flag-IMP3 and Flag-GST were synthesized with an mMESSAGE mMACHINE SP6 kit (Ambion), and the resulting mRNAs (2 μg) were translated in 50 μl of rabbit reticulocyte lysate (Promega). Flag-IMP3 and Flag-GST were purified by immunoprecipitation with anti-Flag antibody. The 3' UTRs of zebrafish *cyclin B1* and *β-globin* mRNAs were amplified by RT-PCR with the following primer sets: for *cyclin B1*, 5'-ttggggttatgctg-3' and 5'-aaaactttaaaaagtttatttgaa-3'; for *β-globin*, 5'-agtctcatcgccaatgaacg-3' and 5'-gcttttaacattattttattgat-3'. The resulting cDNAs were cloned into pGEM T-easy vector (Promega), and DIG-labeled RNA probes were synthesized with T7 RNA polymerase and a DIG RNA labeling mix (Roche). Cross-linking was performed as described previously [9].

2.6. RT-PCR analysis following immunoprecipitation (IP/RT-PCR)

After extraction of RNA from anti-IMP3 immunoprecipitates with Isogen (Nippon Gene), cDNA was synthesized with a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Subsequent PCRs were performed with Taq DNA Polymerase (Ampliqon) and the following primer sets: for *cyclin B1*, 5'-gagggcctttctaagcatctggctgtg-3' and 5'-ttatttgaattcaaatgtacaaacttgc-3'; for *mos*, 5'-tataacctgcgccctttgaccagc-3' and 5'-acatttttgcataaaaaatttagcttcac-3'; for *wee1*, 5'-ttttatccatccaagcaagcgagc-3' and 5'-tttacaaacaaagagttaacaagacc-3'; for *β-actin*, 5'-ggtagttgtctaacaggggagagc-3' and 5'-gttgacttgtcagtgtacagaga-3'.

2.7. Section in situ hybridization and immunostaining

Histological sections were simultaneously analyzed by *in situ* hybridization and immunostaining. Ovaries were fixed with 4% paraformaldehyde in PBS overnight at 4°C, embedded in paraffin, and cut into 12-μm-thick sections. After hybridization with DIG-labeled antisense RNA probes, samples were incubated with a mixture of anti-DIG guinea pig antibody (1:200 dilution) and anti-IMP3 mouse antibody (1:200 dilution) overnight at room temperature. Following treatment with appropriate secondary antibodies conjugated with Alexa 488 for *cyclin B1* mRNA or Alexa 546 for IMP3 (Molecular Probes), sections were observed under a Zeiss LSM 5 LIVE confocal laser microscope.

2.8. Oocyte microinjection

Using a Drummond Nanoject II microinjector, manually isolated full-grown oocytes were injected with 2.3 nl of PBS containing 2.3 ng Flag-IMP3 mRNA, Flag-GST mRNA or no mRNA as a vehicle control. Injected oocytes were incubated in culture medium for 2 h at 26°C and induced to mature. Oocytes were also injected with 2.3 nl of PBS containing 0.08 ng anti-IMP3 antibody or anti-GST antibody as a control

[12] and incubated in culture medium for 30 min at 26°C before inducing maturation.

3. Results

3.1. Identification of novel cyclin B1 mRNA-binding proteins

To isolate proteins bound to *cyclin B1* mRNA, we fractionated ovary extracts by sucrose density gradient ultracentrifugation (Fig. 1A). Cdc2, the catalytic subunit of MPF, which exists as a monomeric form in immature fish and amphibian oocytes except *Xenopus* [1], was found in lighter fractions. In contrast, *cyclin B1* mRNAs were detected in heavier fractions, predominantly in fractions 7 and 8, indicating that *cyclin B1* mRNAs form a large complex in oocytes. Pumilio1 and CPEB, both of which interact with *cyclin B1* mRNA [9], were also abundant in these fractions. Following separation by SDS-PAGE and detection by silver staining of proteins in fractions 7 and 8, nine protein bands were isolated and analyzed by mass spectrometry. Five proteins were identified as candidates for *cyclin B1* mRNA-binding proteins: insulin-like growth factor 2 mRNA-binding protein 3 (IMP3), LSM14 homolog A, DDX6, ELAV-like protein 2 and Zygote arrest protein 1.

We then examined whether the candidate proteins can bind to the 3’ UTR of *cyclin B1* mRNA by UV cross-linking assay (Fig. 1B). Although GST as a control did not interact, IMP3 and ELAV-like protein 2 interacted with the *cyclin B1* 3' UTR but not with the control *β-globin* 3' UTR. Unfortunately, we were unable to obtain clear results showing interaction of *cyclin B1* mRNA with the three other candidate proteins. Since IMP3 was also detected in *cyclin B1* mRNA aggregates isolated by laser capture micro-dissection (our unpublished result), we thus decided to focus on IMP3 in this study.

3.2. Detection of IMP3 in oocytes

An anti-IMP3 antibody recognized a 69-kDa protein in both immature and mature oocytes (Fig. 1C). The molecular mass of *Xenopus* IMP3 is 69 kDa [14] and that estimated from the cDNA sequence of

zebrafish IMP3 is 64 kDa. The anti-IMP3-positive 69-kDa protein was enriched in fractions 7 and 8, similar to *cyclin B1* mRNA, Pumilio1 and CPEB (Fig. 1A). We therefore concluded that the antibody recognizes IMP3. The protein levels of IMP3 remained constant during oocyte maturation, like γ-tubulin as a loading control (Fig. 1D). As in the case of *Xenopus* [12], Pumilio1, CPEB and MAP kinase were phosphorylated during maturation and CPEB was degraded in mature oocytes. Since *Xenopus* IMP3 is phosphorylated during oocyte maturation [15], we examined incorporation of ^{32}P into zebrafish IMP3 during oocyte maturation by injection into oocytes or incubation of oocyte extracts with [^{32}P]ATP. However, IMP3 was not labeled with ^{32}P. We then examined the phosphorylation state by Phos-tag SDS-PAGE (Fig. 1E). Retardation in electrophoretic mobility of zebrafish IMP3 was observed in both immature and mature oocytes, and the retarded bands disappeared when treated with λ-phosphatase. These findings suggest that zebrafish IMP3 is already phosphorylated in immature oocytes and the phosphorylation state is unchanged during oocyte maturation.

3.3. Interaction between endogenous IMP3 and cyclin B1 mRNA

Interaction between IMP3 and *cyclin B1* mRNA *in vivo* was investigated by IP/RT-PCR (Fig. 2A). Anti-IMP3 immunoprecipitates from immature oocytes contained *cyclin B1* mRNA, but those from mature oocytes did not. Anti-IMP3 immunoprecipitates, however, did not contain *mos* and *wee1* mRNAs, which are polyadenylated during *Xenopus* oocyte maturation, like *cyclin B1* mRNA [4]. In contrast, anti-Pumilio1 immunoprecipitates from both immature and mature oocytes contained *cyclin B1* mRNA but not *mos* and *wee1* mRNAs, consistent with our previous finding in *Xenopus* oocytes [12]. All of the immunoprecipitates examined did not contain *β-actin* mRNA as a control. These results indicate that IMP3 specifically interacts with *cyclin B1* mRNA in immature oocytes but dissociates from it in mature oocytes, while Pumilio1 remains associated with the mRNA throughout oocyte maturation.

3.4. Interaction of IMP3 with Pumilio1 or CPEB

We examined the interaction between IMP3 and Pumilio1 by co-immunoprecipitation assay (Fig. 2B). Anti-IMP3 immunoprecipitates from immature oocyte extracts contained Pumilio1 and, conversely, anti-Pumilio1 immunoprecipitates contained IMP3. However, interaction between IMP3 and Pumilio1 was not detected in mature oocyte extracts, in agreement with the results of IP/RT-PCR indicating that IMP3 dissociates from *cyclin B1* mRNA but that Pumilio1 remains associated in mature oocytes (Fig. 2A). In addition, the interaction between Pumilio1 and IMP3 was disrupted by RNase treatment. These results revealed that the interaction of IMP3 and Pumilio1 in immature oocytes is indirect and dependent on the presence of *cyclin B1* mRNA.

In *Xenopus*, Pumilio1 and CPEB form a complex directly via protein-protein interaction [12,13]. We then investigated the interaction between CPEB and IMP3 (Fig. 2C). Since CPEB overlapped with immunoglobulins used for immunoprecipitation, it was difficult to detect CPEB in anti-IMP3 immunoprecipitates. We thus examined IMP3 in anti-CPEB immunoprecipitates and found that IMP3 indirectly interacts with CPEB through *cyclin B1* mRNA in immature oocytes, similar to the interaction between IMP3 and Pumilio1.

3.5. Subcellular localization of IMP3 in oocytes

We examined the localization of *cyclin B1* mRNA and IMP3 by simultaneous analyses of *in situ* hybridization and immunostaining (Fig. 3). Double staining of the identical sections demonstrated co-localization of *cyclin B1* mRNA and IMP3 in the animal polar cytoplasm of immature oocytes. In mature oocytes, however, IMP3 did not exhibit any subcellular localization (data not shown), in harmony with the dispersion of *cyclin B1* mRNA in the cytoplasm [8,11].

3.6. Effects of IMP3 overexpression and anti-IMP3 antibody injection on oocyte maturation

To know the involvement of IMP3 in oocyte maturation, we performed IMP3 overexpression experiments. Oocytes were microinjected with Flag-IMP3 mRNA, Flag-GST mRNA or PBS and induced to mature. Protein expression from the injected mRNAs was confirmed by immunoblotting (Supplementary Figure 2B). Oocytes overexpressing IMP3 underwent GVBD at a significantly later timing than did control oocytes injected with Flag-GST mRNA or PBS (Fig. 4A and B).

We also injected anti-IMP3 antibody into oocytes. Although anti-GST antibody or PBS as controls had no effect on oocyte maturation, the injection of anti-IMP3 antibody delayed the progression of maturation (Fig. 4C and D), similar to the results of IMP3 overexpression experiments.

4. Discussion

We identified IMP3 as a novel *cyclin B1* mRNA-binding protein. IMP3 was described as a protein bound to the vegetal localization element (VLE) in the 3'UTR of *Vg1* mRNA that localizes to the vegetal cortex of *Xenopus* oocytes (thereby also called *Vg1* RNA-binding protein) [14]. Since the 3' UTR of zebrafish *cyclin B1* mRNA harbors an E2 motif (UUCAC), a *cis*-acting element in the VLE [16], it is plausible that IMP3 binds to *cyclin B1* mRNA via this motif in zebrafish oocytes. In contrast, *Xenopus cyclin B1* mRNA lacks an E2 motif in its 3' UTR and does not localize to the vegetal pole of *Xenopus* oocytes. Therefore, IMP3 targets *cyclin B1* mRNA in zebrafish oocytes but not in *Xenopus* oocytes. It is also notable that unlike *Xenopus*, in which *Vg1* mRNA localizes to the vegetal pole, zebrafish *Vg1* mRNA localizes to the animal polar cytoplasm in immature oocytes [17], regardless of the lack of an E2 motif. In addition, zebrafish *nanos1* mRNA does not localize to the animal pole (but localizes to the vegetal pole) during early oogenesis [18], whereas it contains two E2 motifs in the 3' UTR. Consequently, the presence of an E2 motif is neither necessary nor sufficient for the localization of mRNAs to the animal polar cytoplasm in immature zebrafish oocytes. Further studies are required to understand a general mechanism that localizes different mRNAs to the animal polar cytoplasm in zebrafish oocytes.

IMP3 is phosphorylated by Erk2/MAP kinase on S402 that exists in the linker separating the second and third hnRNP K-homology (KH) domains, resulting in the cortical release of *Vg1* mRNA during *Xenopus* oocyte maturation [15]. The minimum consensus sequence phosphorylated by Erk2/MAP kinase is S/TP [19]; however, the corresponding serine residue followed by proline is only conserved in *X. laevis* and *X. tropicalis* IMP3 and is absent in other vertebrate homologs including zebrafish IMP3 (Supplementary Figure 3). Consistent with this, we found that zebrafish IMP3 is not newly phosphorylated, despite the fact that MAP kinase is activated after hormonal stimulation (Fig. 1D). It is highly likely that zebrafish IMP3 is already phosphorylated in immature oocytes in the absence of MAP kinase activation and that the phosphorylation state is constant during oocyte maturation (Fig. 1E). Irrespective of the steady phosphorylation state, IMP3 dissociates from *cyclin B1* mRNA in mature oocytes (Fig. 2). A chemical modification of IMP3 other than phosphorylation might cause the release of IMP3 from the *cyclin B1* mRNA. Alternatively, a chemical modification, including phosphorylation, of a protein(s) that binds directly to IMP3 might weaken the interaction between IMP3 and the mRNA.

In immature *Xenopus* oocytes, IMP3 is bound to *Vg1* mRNA that localizes to the vegetal cortex [14], and its phosphorylation by MAP kinase during oocyte maturation induces cortical detachment and solubilization of *Vg1* mRNA [15]. While these findings suggest that IMP3 plays a role in the localization of *Vg1* mRNA, the involvement of IMP3 in the translational repression or activation of *Vg1* mRNA in *Xenopus* oocytes is ambiguous. In fact, the translation of *Vg1* mRNA has been reported to be repressed by ElrB (HuB) in growing oocytes until stage IV but activated thereafter even in the presence of IMP3 [20]. To gain insight into the function of IMP3 in zebrafish oocytes, we overexpressed IMP3 in oocytes to enhance its function. Overexpression of IMP3 retarded the progression of oocyte maturation (Fig. 4A and B), suggesting that IMP3 functions as a repressor of *cyclin B1* mRNA translation. Microinjection of anti-IMP3 antibody also caused a delay in the timing of oocyte maturation (Fig. 4C and D). The injected antibody might prevent conformational changes of IMP3 that are required for its dissociation from *cyclin B1*

mRNA, thereby resulting in a delay in the translational activation of mRNA.

Although the results from overexpression and antibody injection experiments suggest that IMP3 represses the translation of *cyclin B1* mRNA in immature oocytes, we need to clarify its actual function. Recently, we have established an *in vivo* reporter gene assay system in zebrafish, in which reporter mRNAs can precisely mimic the behavior of endogenous *cyclin B1* mRNA and their timings of translational activation during oocyte maturation can be visualized in real time [10,11]. Examination of the behavior of reporter mRNAs with mutations in the VLE in this experimental system should provide a better understanding of the function of IMP3 in the translational regulation of *cyclin B1* mRNA.

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Figure legends

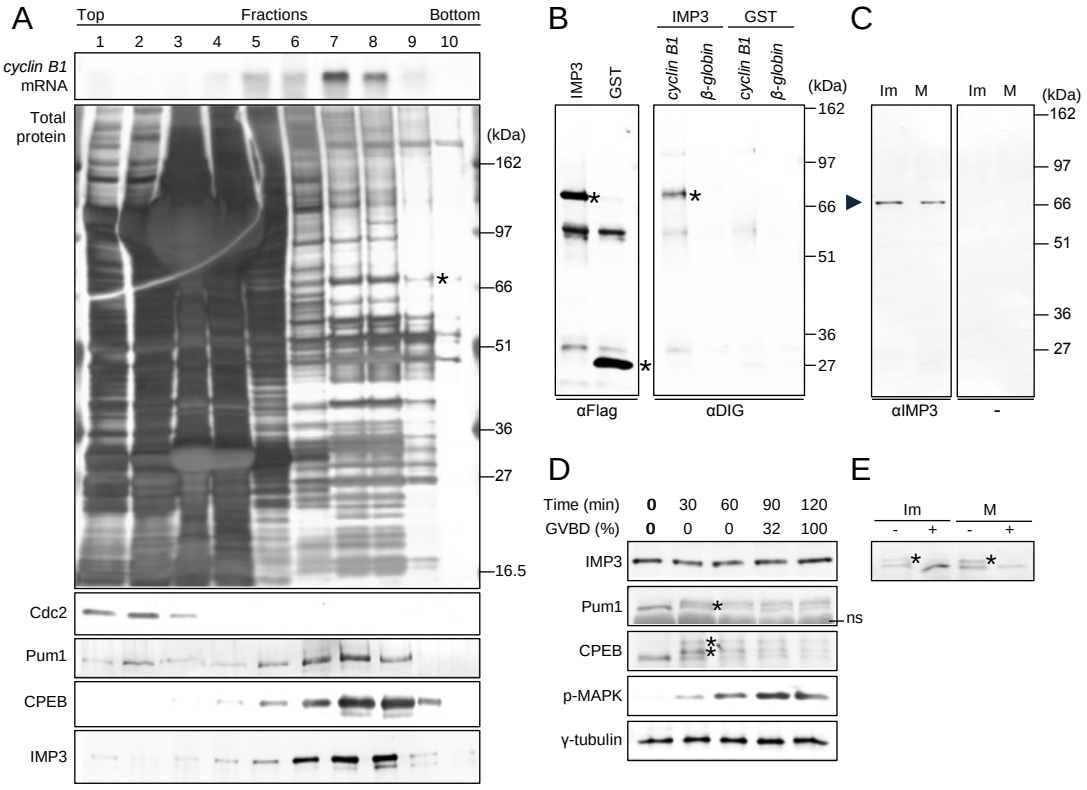
Fig. 1. Identification of IMP3 as a *cyclin B1* mRNA-binding protein. (A) Fractionation by sucrose density gradient ultracentrifugation. *cyclin B1* mRNA, total protein, Cdc2, Pumilio1 (Pum1), CPEB and IMP3 in each fraction are shown. (B) UV cross-linking assay. Flag-tagged IMP3 and GST shown by anti-Flag immunoblotting (αFlag) were incubated with DIG-labeled *cyclin B1* or *β-globin* 3' UTR, and the bound probes were detected with anti-DIG immunoblotting (αDIG). Asterisks indicate specific signals. (C) Immunoblotting of immature (Im) and mature (M) oocyte extracts with anti-IMP3 antibody (αIMP3) or without a primary antibody (-). Arrowhead shows the anti-IMP3-positive 69-kDa protein. (D) Hormone-treated oocytes were extracted at indicated times and analyzed by anti-IMP3, anti-Pumilio1 (Pum1), anti-CPEB, anti-phospho-MAP kinase (p-MAPK) or anti-γ-tubulin immunoblotting. Asterisks indicate phosphorylated Pumilio1 and CPEB. Non-specific bands on the anti-Pum1 immunoblot are marked as ns. (E) Anti-IMP3 immunoprecipitates from immature (Im) and mature (M) oocyte extracts were treated with (+) or without (-) λ-phosphatase and analyzed by phos-tag SDS-PAGE followed by anti-IMP3 immunoblotting. Asterisks indicate phosphorylated IMP3.

Fig. 2. Interaction of IMP3 with *cyclin B1* mRNA, Pumilio1 and CPEB. (A) Immature and mature oocyte extracts were subjected to immunoprecipitation (IP) with anti-Pumilio1 (αPum1), anti-IMP3 (αIMP3) or without an antibody (-). Indicated mRNAs in the precipitates and extracts before IP (initial) were detected by RT-PCR. (B) Immature (Im) and mature (M) oocyte extracts were treated with (+) or without (-) RNase A and immunoprecipitated with anti-Pumilio1 (αPum1), anti-IMP3 (αIMP3) or without an antibody (-). Pumilio1 (Pum1) and IMP3 in the precipitates and crude extracts (crude) were detected by immunoblotting. (C) Experiments similar to those in (B) using anti-CPEB antibody for immunoprecipitation.

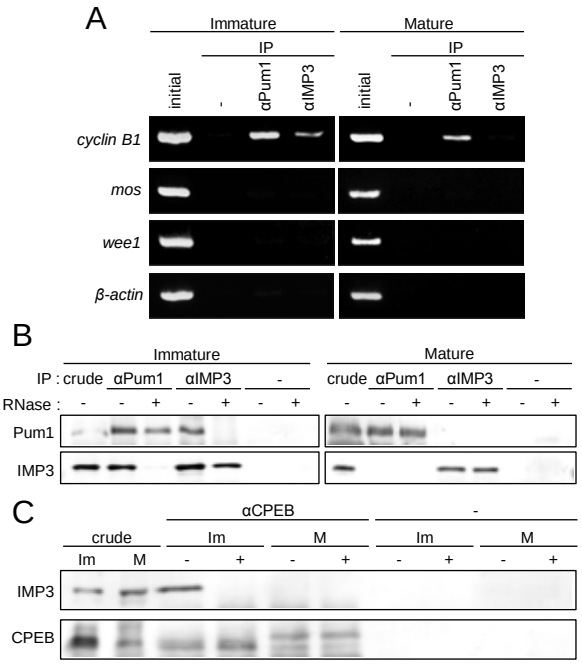
Fig. 3. Subcellular localization of IMP3 and *cyclin B1* mRNA in immature oocytes. *In situ* hybridization of *cyclin B1* mRNA (green), immunostaining of IMP3 (red), and merged images are shown. The boxed regions in panel A are magnified in panel B. Results of a control experiment without primary antibodies are shown in panel C, in which the egg chorion is outlined by broken lines. EC, egg chorion; FC, follicle cell; MP, micropyle. Bars, 100 μm (A), 50 μm (B and C). See also Supplementary Figure 1 for the general morphology of zebrafish oocytes.

Fig. 4. Effects of IMP3 overexpression and anti-IMP3 antibody injection on oocyte maturation. (A) Time course of GVBD in oocytes injected with Flag-IMP3, Flag-GST or PBS. (B) Percentage of GVBD in the Flag-IMP3- or Flag-GST- injected oocytes at the time when the PBS-injected oocytes undergo ~50% GVBD. Values are means ± S.D. for three independent experiments. Statistical analysis was performed by Student's *t*-test (**P* < 0.05, ***P* < 0.005, ****P* < 0.001). (C, D) Anti-IMP3 antibody injection experiments. Legends are similar to (A) and (B).

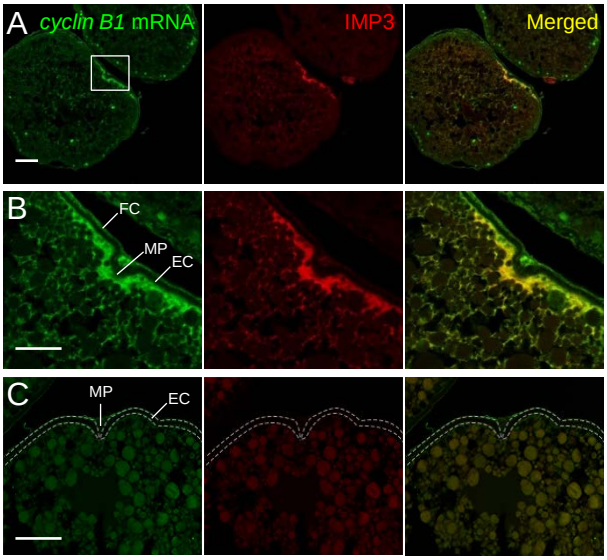
Takahashi et al. Figure 1



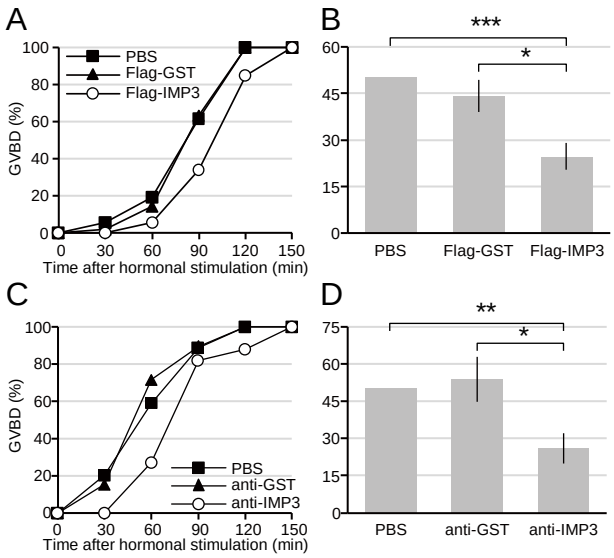
Takahashi et al. Figure 2



Takahashi et al. Figure 3



Takahashi et al. Figure 4



Appendix A. Supplementary data

Supplementary Figure 1. Morphology of the animal polar region of immature zebrafish oocytes. (A) An Epon thick section stained with toluidine blue. Full-grown oocytes were prefixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed with 1% OsO_4 in the same buffer, dehydrated in acetone, and embedded in Epon. Sections (1-2 μm thick) were stained with 0.1% toluidine blue in 1% sodium borate. (B) *In situ* hybridization of *cyclin B1* mRNA. Oocytes were fixed with 4% paraformaldehyde in PBS, dehydrated in ethanol, and embedded in paraffin. Sections (12 μm thick) were hybridized with DIG-labeled antisense *cyclin B1* probes, and the bound probes were detected with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche). Purple signals show *cyclin B1* mRNAs localized to the animal polar cytoplasm. CA, cortical alveolus; EC, egg chorion; FC, follicle cell; GV, germinal vesicle; MP, micropyle; YG, yolk granule. Scale, 50 μm.

Supplementary Figure 2. Immunoblots of zebrafish oocyte extracts. (A) Specificity of anti-CPEB monoclonal antibodies. Extracts from immature (Im) and mature (M) zebrafish oocytes were separated by SDS-PAGE and immunoblotted with anti-goldfish CPEB monoclonal antibody (αGFCPEB 3D3 or 9G3) or without primary antibody (-). Phosphorylated forms of CPEB in mature oocyte extracts are not clearly shown in the blots. (B) Overexpression of IMP3 and GST in immature oocytes. Oocytes injected with Flag-IMP3 mRNA, Flag-GST mRNA or PBS were examined by immunoblotting with anti-Flag antibody (αFlag) to detect the proteins expressed from each mRNA.

Supplementary Figure 3. Alignment of a sequence around S402 and P403 in the frog *Xenopus laevis* IMP3 (GeneBank accession: NP_001081752) with the corresponding sequences in the frog *X. tropicalis* (NP_001017139), human *Homo sapiens* (NP_006538), mouse *Mus musculus* (NP_076159), chick *Gallus gallus* (NP_001006359), turtle *Pelodiscus sinensis* (XP_006138418) and zebrafish *Danio rerio*

(NP_571566) IMP3. S402 is phosphorylated by MAP kinase during oocyte maturation in *X. laevis*. Note that the minimum consensus sequence phosphorylated by MAP kinase (SP, shown in red) is conserved in *X. laevis* and *X. tropicalis* IMP3 but not in other homologs. KH, hnRNP K-homology domain.

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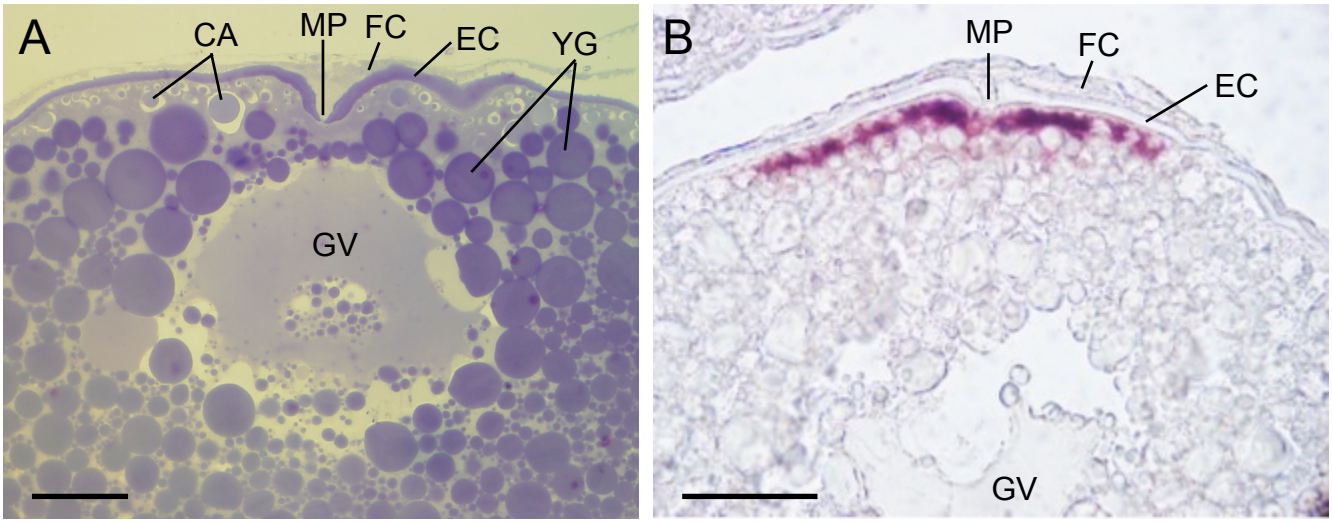

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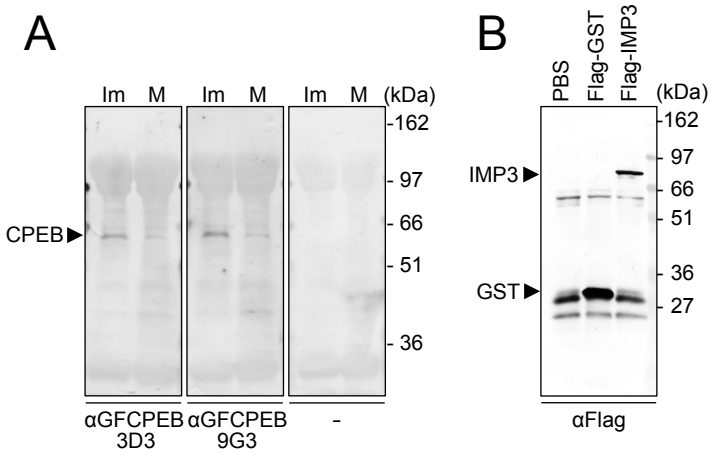
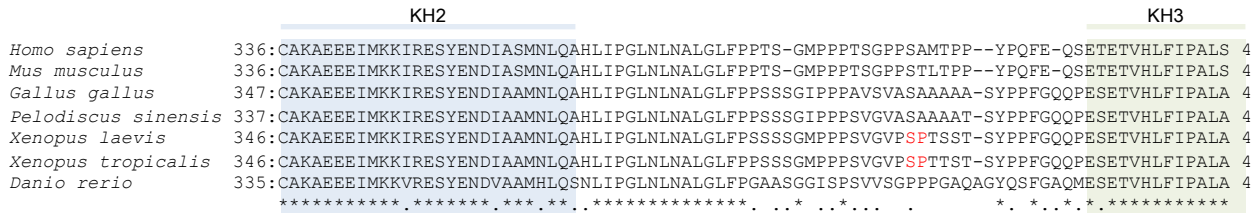
Takahashi et al. Supplementary Figure 3