Mechanisms regulating the translation and localization of *cyclin B1* mRNA in zebrafish oocytes

（ゼブラフィッシュ卵母細胞におけるサイクリン B1 mRNA の翻訳と局在を制御する機構）

A DISSERTATION

Submitted to the Graduate School of Life Science,

Hokkaido University

in partial fulfillment of the requirements for the degree

DOCTOR OF LIFE SCIENCE

by

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March, 2017

Contents

Acknowledgments

I would like to express my sincere gratitude to Professor Masakane Yamashita for his mentorship throughout the course of my studies. I am deeply grateful to Professors Kazuma Tanaka, Yoshinao Katsu and Asato Kuroiwa for reviewing the manuscript and their critical advices to improve the thesis. I would like to sincerely thank Associate Professors Tomoya Kotani and Katsueki Ogiwara for their helpful technical advices and discussions to progress this study. I am also grateful to Emeritus Professors Takayuki Takahashi and Takashi Shimizu and Associate Professor Atsushi P. Kimura for their helpful advices and encouragement throughout this study. I also heartily thank Drs. Ryoma Ota and Kyota Yasuda and Mr. Ryuichi Satoh for their technical instructions and valuable advices. I would also like to thank Ms. Seiko Oka of the Global Facility Center, Hokkaido University for mass spectrometry analysis. I also thank Kana Ishii for producing anti-Staufen1 antibodies. Special thanks are also due to all the members of the Yamashita laboratory and the laboratories of Reproductive and Developmental Biology for their help, discussion, cooperation and friendship in the daily life.

Abbreviations

ARE	AU-rich element
DIG	digoxigenin
EB	extraction buffer
EB1	End-binding protein 1
GP	Guinea pig
GST	glutathione-S-transferase
GV	germinal vesicle
GVBD	GV breakdown
IMP3	Insulin-like growth factor 2 mRNA-binding protein 3
IP	immunoprecipitation
KH domain	hnRNP K-homology domain
M	Mouse
MAPK	mitogen-activated protein kinase
MIH	maturation-inducing hormone
MPF	maturation/M-phase-promoting factor
mRNP	messenger ribonucleoprotein
ORF	open-reading frame
PBE	Pumilio-binding element
PFA	paraformaldehyde
Pum1	Pumilio1
qRT-PCR	quantitative real-time RT–PCR
RT-PCR	reverse transcription-polymerase chain reaction
Stau1	Staufen1
Vg1RBP	Vgl RNA-binding protein
VLE	vg1/vegetal localization element

General Introduction

In many animal species, full-grown oocytes after accumulation of yolk and other materials stop their meiotic cell cycle at prophase I and unable to be fertilized, thereby being called immature oocytes. In response to maturation-inducing hormone (MIH), immature oocytes resume the meiosis, proceed to metaphase II and become mature fertilizable oocytes. This process is called oocyte maturation, which is accompanied by such morphological changes as germinal vesicle breakdown (GVBD), chromosome condensation, spindle formation and the first polar body extrusion. These events are driven by the kinase activity of maturation-promoting factor (MPF), a protein complex consisting of the catalytic subunit Cdc2 and the regulatory subunit Cyclin B1. Except for African clawed frog (*Xenopus laevis*) in which Cyclin B-bound inactive Cdc2 (pre-MPF) exists in immature oocytes, Cyclin B1 protein is absent in immature oocytes of fish and amphibians, because translation of *cyclin B1* mRNA is completely repressed in these species. MIH stimulation induces translational activation of dormant maternal mRNAs including *cyclin B1* mRNA, resulting in the formation of MPF and the completion of oocyte maturation. In particular, microinjection of Cyclin B1 protein into full-grown oocytes of Japanese brown frog (*Rana japonica*), goldfish (*Carassius auratus*) or zebrafish (*Danio rerio*), in which pre-MPF is absent, induces MPF activation and subsequent GVBD without MIH stimulation; therefore, translational regulation of *cyclin B1* mRNA is critical to initiate the process of oocyte maturation in these species (Katsu et al., 1993; Tanaka and Yamashita, 1995; Kondo et al., 1997; Ihara et al., 1998; Yamashita, 1998, 2000; Nagahama and Yamashita, 2008).

Since gene transcription is quiescent until the midblastula transition, protein synthesis during oocyte maturation and the early phase of embryonic development depends solely on translation of maternal mRNAs which are synthesized during oocyte growth and stored as a dormant form. Therefore the mechanisms to regulate both translational repression and

activation of mRNAs stored in the oocytes have important roles to assure the normal progression of oocyte maturation and early embryogenesis. Such translational regulation is mainly achieved by cytoplasmic polyadenylation, which is controlled by the activity of cytoplasmic polyadenylation element (CPE)-binding protein (CPEB) and its common co-factors including Symplekin (a scaffolding protein), GLD-2 (a poly(A) polymerase) and PARN (a poly(A) ribonuclease). Although both GLD-2 and PARN are active, poly(A) tail lengths of the CPE-containing mRNAs remain short, because the ribonuclease activity of PARN surpasses the polymerase activity of GLD-2 on the translationally repressed mRNAs. Hormonal stimulation activates a signal transduction pathway that phosphorylates CPEB, which causes the dissociation of PARN from the ribonucleoprotein complex, leading to translational activation (Sheets et al., 1994; Ballantyne et al., 1997; de Moor and Richter, 1997, 1999; Barkoff et al., 2000; Charlesworth et al., 2000; Mendez and Richter, 2001; Barnard et al., 2004; Kim and Richter, 2006; Richter, 2007; Piqué et al., 2008; Radford et al., 2008). After hormonal stimulation, however, proteins involved in meiotic cell cycle progression must be synthesized at a timing specific to each protein. For example, the strict temporal order of *mos*, *cyclin B1* and *wee1* mRNA translation is important to ensure the progression of oocyte maturation (Murakami and Vande Woude, 1998; Nakajo et al., 2000; Nebreda and Ferby, 2000; Castro et al., 2001; Haccard and Jessus, 2006; Arumugam et al., 2010). The precise translational timings are regulated by CPEB in cooperation with certain partners; Musashi regulates Mos synthesis by binding to Musashi-binding element within the 3' untranslated region (UTR) of *mos* mRNA (Charlesworth et al., 2002, 2006), translation of *cyclin B1* mRNA is regulated by Pumilio1 (Pum1) that binds to Pumilio-binding element (PBE) within the 3' UTR, (Nakahata et al., 2001, 2003; Ota et al., 2011a), and the translational control sequence (TCS) found in 3' UTR of *mos* and *wee1* mRNAs is recognized by Zar proteins, which contribute to their translational repression and activation (Wang et al., 2008; Charlesworth et al., 2012; Yamamoto et al., 2013).

While all of the mechanisms of translational regulation described above were revealed by using *Xenopus* as a model organism, zebrafish *cyclin B1* mRNA is also regulated by the CPE-mediated cytoplasmic polyadenylation (Zhang and Sheets, 2009). In zebrafish oocytes, dormant *cyclin B1* mRNA aggregates at the animal polar cytoplasm beneath the micropyle, an entry point of sperm into fish oocytes. After MIH stimulation, the aggregation of *cyclin B1* mRNA disperses prior to their translational activation, leading to oocyte maturation. Dispersion of *cyclin B1* mRNA from the animal pole is caused by treatment with cytochalasin B, an inhibitor of actin polymerization, which activates translation of *cyclin B1* mRNA without MIH stimulation (Kondo et al., 2001). Indeed, the meshwork of microfilaments beneath the animal pole is reconstructed in response to MIH stimulation (Nukada et al., 2015); therefore, the results of these studies indicate that microfilaments play important roles in the localization and translational regulation of *cyclin B1* mRNA in zebrafish oocytes.

To further understand the mechanisms of translational regulation of *cyclin B1* mRNA, Yasuda et al. developed a novel experimental system in which reporter mRNAs can precisely mimic the behavior of endogenous *cyclin B1* mRNAs in zebrafish oocytes and showed that an unidentified protein(s) which binds to a *cis*-element in the open-reading frame (ORF) of *cyclin B1* mRNA is required for mRNA localization and temporal regulation of translational activation (Yasuda et al., 2010, 2013). It is notable that mutation in the *cis*-element sequence disrupted aggregation of *cyclin B1* mRNA and caused precocious translational activation after MIH stimulation; however, translational repression before MIH stimulation was maintained even in the lack of the *cis*-element and its *trans*-acting protein(s). Using this experimental system, Kotani et al. showed that *cyclin B1* mRNA forms messenger ribonucleoprotein (mRNP) granules and demonstrated that Pum1 that binds to 3’ UTR of *cyclin B1* mRNA contributes to the formation of *cyclin B1* mRNP granules. Mutations in the PBE sequence disrupted association with Pum1 and impaired the

formation of mRNP granules and, consequently, caused precocious translational activation after MIH stimulation. These results indicate that Pum1 regulates the timing of translational activation of *cyclin B1* mRNA through formation and disassembly of mRNP granules. As in the case of the *cis*-element in the ORF, however, translational repression before MIH stimulation was maintained even in the absence of Pum1, meaning that Pum1 is critical for fine-tuning of the timing of translational activation after MIH stimulation, and that the other translational regulators including CPEB play a fundamental role in translational repression in immature oocytes. Therefore, molecular mechanisms regulating post-transcriptional events before MIH stimulation, including mRNA transport and localization to the animal polar cytoplasm and translational repression and activation in immature and maturing oocytes, remain to be elucidated.

To address these issues, in Chapter I, I performed sucrose density gradient ultracentrifugation followed by mass spectrometry analysis and identified an RNA-binding protein, insulin-like growth factor 2 mRNA-binding protein 3 (IMP3) as a possible regulator of mRNAs. IMP3 is expressed in immature oocytes and its protein content and phosphorylation state remain constant through oocyte maturation. IMP3 binds to *cyclin B1* mRNA and is localized at the animal polar cytoplasm in immature oocytes, but its interaction with *cyclin B1* mRNA is disrupted in mature oocytes. Flag-tagged IMP3 which was overexpressed by injecting mRNA co-localized with endogenous *cyclin B1* mRNA at the animal polar cytoplasm and caused delay in the translation of *cyclin B1* mRNA and progression of oocyte maturation. On the basis of these results, I propose that IMP3 represses the translation of *cyclin B1* mRNA besides CPEB and Pum1 in immature zebrafish oocytes.

IMP3 was initially described as Vgl RNA-binding protein (Vg1RBP), a protein bound to *vg1* mRNA that is localized to the vegetal cortex of *Xenopus* oocytes along the mRNA localization pathway called late (Vg1) pathway (Mowry and Melton, 1992; Schwartz et al., 1992). In zebrafish oocytes, *vg1* mRNA is localized to the animal polar cytoplasm similarly to

cyclin B1 mRNA, and I found that IMP3 interacts with *vg1* mRNA, as well as *cyclin B1* mRNA. These results suggest that common proteins are involved in the localization machinery of *cyclin B1* mRNA to the animal pole in zebrafish oocytes and *Vg1* mRNA to the vegetal pole in *Xenopus* oocytes, and this provides a key insight into the unknown mechanisms for *cyclin B1* mRNA localization to the animal polar cytoplasm in zebrafish oocytes.

In Chapter II, I analyzed zebrafish Staufen1 (Stau1) protein, because this protein is known to play a key role in localization of *vg1* mRNA in association with kinesin motor proteins in *Xenopus* oocytes. Zebrafish Stau1 was predominantly expressed in oocytes of stages I to III, but its expression level decreases thereafter, suggesting that Stau1 mainly functions in young oocytes. I also showed that zebrafish Stau1 interacts with *cyclin B1* mRNA throughout oocyte growth and maturation. The section *in situ* hybridization and immunohistochemistry analyses showed that both *cyclin B1* mRNA and Stau1 protein distribute throughout the cytoplasm in young oocytes, but co-localized to the animal polar cytoplasm of stage III or later oocytes. Transportation of *gfp-cyclin B1* reporter mRNA to the animal pole was inhibited by disruption of microtubules and in the presence of antibodies against Stau1 or Kinesin1. These results suggest that zebrafish *cyclin B1* mRNA is transported toward the animal polar cytoplasm depending on both Staufen1 and Kinesin1 along microtubules, in a similar way to the *vg1* mRNA in *Xenopus* oocytes. In addition, I demonstrated that the reporter mRNAs with an element required for the vegetal localization in *Xenopus* oocytes is transported to the animal pole in zebrafish oocytes. I also showed that the plus ends of microtubules which face the vegetal pole in *Xenopus* oocytes face the animal pole in zebrafish oocytes. Based on these results, I propose that *cyclin B1* mRNA is transported to the animal polar cytoplasm by a common mRNA transport machinery in zebrafish and *Xenopus* oocytes, although its transport directions are opposite probably due to different organizations of microtubules in oocytes.

In this thesis, I focus on the molecular mechanisms underlying the translational regulation and localization of *cyclin B1* mRNA throughout oocyte growth and maturation in zebrafish by biochemically and functionally characterizing IMP3 in Chapter I and Stau1 in Chapter II. I believe that these studies provide a valuable perspective on the comprehensive understanding of the post-transcriptional regulation of *cyclin B1* mRNA in oocytes.

Chapter I

Involvement of IMP3 in translational repression of *cyclin B1* mRNA during oocyte maturation

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 CPE and CPEB, Pum1 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. I report that IMP3, which was initially described as a protein bound to *vg1* mRNA localized to the vegetal pole of *Xenopus* oocytes, binds to and co-localizes with *cyclin B1* mRNA in immature zebrafish oocytes. Although protein content and phosphorylation state of IMP3 remain unchanged during oocyte maturation, IMP3 dissociates from *cyclin B1* mRNA after hormonal stimulation. In accordance with this, the mRNA-dependent interactions of IMP3 with Pum1 and CPEB in immature oocytes were disrupted in mature oocytes. The overexpressed IMP3 proteins were accumulated at the animal polar cytoplasm and caused delay in the polyadenylation of *cyclin B1* mRNA, synthesis of Cyclin B1 protein and progression of oocyte maturation. On the basis of these results, I 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.

Introduction

In many animal species, the meiotic cell cycle of immature oocytes is arrested at prophase I, even when the oocytes have fully grown after accumulation of substances necessary for early embryogenesis. These oocytes resume the meiosis in response to MIH and become mature fertilizable oocytes after reaching metaphase II. This process called oocyte maturation includes morphological changes such as GVBD, chromosome condensation and spindle formation, all of which are caused by the kinase activity of MPF, a protein complex consisting of the catalytic subunit Cdc2 and regulatory subunit Cyclin B1. Since Cyclin B1 protein is absent in immature oocytes of fish and amphibians except for *Xenopus laevis*, its *de novo* synthesis is essential for the initiation of oocyte maturation in these species (Yamashita, 1998, 2000; Nagahama and Yamashita, 2008).

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 (Sheets et al., 1994; Ballantyne et al., 1997; de Moor and Richter, 1997), which is mainly regulated by the CPEB that resides in the 3' UTR. Besides CPEB, however, other RNA-binding proteins specific to each target mRNA are required for assuring the timings of translational activation (Radford et al., 2008; MacNicol and MacNicol, 2010).

Fish oocytes are characterized by the micropyle, a sperm entry hole on the egg chorion at the animal pole (cf. Fig. 4A), while amphibian (*Xenopus*) oocytes lack the micropyle. Although both zebrafish and *Xenopus cyclin B1* mRNAs are polyadenylated and translationally activated during oocyte maturation (Zhang and Sheets, 2009), their localization in immature oocytes is different. In zebrafish, dormant *cyclin B1* mRNAs aggregate and localize to the animal polar cytoplasm beneath the micropyle (cf. Fig. 4B),

whereas similar aggregation and localization have not been reported for *Xenopus cyclin B1* mRNA. After MIH 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 (Kondo et al., 1997, 2001). The aggregation of *cyclin B1* mRNA requires Pum1 (Kotani et al., 2013), which also regulates translation of *cyclin B1* mRNA during *Xenopus* oocyte maturation (Nakahata et al., 2001, 2003; Ota et al., 2011a). In addition, an unidentified protein(s) bound to a *cis*-element consisting of 9 nucleotides in the coding region of *cyclin B1* mRNA is required for the mRNA localization and temporal regulation of translational activation (Yasuda et al., 2010, 2013). These studies have uncovered key mechanisms of translational control of *cyclin B1* mRNA; 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.

To address this issue, I identified several mRNA-binding proteins and examined IMP3. IMP3 was initially described as a Vg1RBP which specifically binds to the vg1/vegetal localization element (VLE) of *vg1* mRNA 3' UTR in *Xenopus* oocytes. Vg1RBP is localized to the vegetal cortex of oocytes along with *vg1* mRNA (Mowry and Melton, 1992; Schwartz et al., 1992) and phosphorylated by mitogen-activated protein kinase (MAPK) during oocyte maturation, which allows *vg1* mRNA to be released from the vegetal cortex (Git et al., 2009). All the vertebrate homologs of IMP3 contain two RNA recognition motifs and four hnRNP K-homology (KH) domains consisting of two pairs of didomains that are responsible for self-association and RNA-binding (Git and Standart, 2002). These homologs exist in various cell types and play important roles in post-transcriptional regulation including mRNA transport (Deshler et al., 1998), translational repression (Nielsen et al., 1999), translational activation (Liao et al., 2005), and mRNA stabilization (Vikesaa et al., 2006). Besides the function as a post-transcriptional regulator, IMP3 is

strikingly investigated as an oncofetal protein because of its predominant expression during embryogenesis and its remarkable upregulation in some tumors (Yaniv and Yisraeli, 2002; Lederer et al., 2014). In this study, I showed the involvement of IMP3 in translational regulation of *cyclin B1* mRNA in zebrafish oocytes. The results of this study will provide an insight into the function of IMP3 and the translational regulation of dormant mRNAs in oocytes.

Materials and methods

Oocyte culture and extraction

All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University (permission No. 13-0099). Full-grown oocytes were manually isolated from ovaries and placed in zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2) (Kondo et al., 1997). Oocyte maturation was induced by incubating the oocytes in culture medium (90% Leibovitz's L-15, 0.5% bovine serum albumin, 100 μg/ml gentamycin; pH 9.0) (Seki et al., 2008; Nair et al., 2013) containing 1 μg/ml 17α,20β-dihydroxy-4-pregnen-3-one, an MIH in fish. For preparing oocyte extracts, oocytes were washed three times with ice-cold extraction buffer (EB: 100mM β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 100 μM PMSF, 3 μg/mL leupeptin, pH7.5). After the final wash, the oocytes were homogenized with new EB and centrifuged at 5,000 g for 5 min at 4°C, and the supernatants were diluted with EB and used as oocyte extracts (1 μl/oocyte). For RNA analysis, RNasin Plus RNase inhibitor (100 units/ml at the final concentration, Promega) was added to extraction buffer.

Production of recombinant proteins and antibodies

To produce a full-length IMP3 protein with a Flag tag at the N terminus (Flag-IMP3), an ORF of zebrafish IMP3 (NM_131491.2) was amplified with a primer set of IMP3_ORF_F and IMP3_ORF_R (Table 1) which introduces *Cla*I and *Xho*I sites. The PCR product was cloned into pGEM-T easy vector (Promega), digested with *Cla*I and *Xho*I, and ligated into pCS2+FT-N (Ota et al., 2011b).

To produce a truncated IMP3 protein 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 of IMP3_ex_F and

IMP3_ex_R (Table 1). 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, as described previously (Hirai et al., 1992). The purified GST-IMP3 was dialysed against 1 mM HEPES (pH 7.0), lyophilized, and they were injected into mice to produce polyclonal antibodies, according to the procedures described previously (Yamashita et al., 1991). IMP3-His protein was electroblotted onto an Immobilon membrane and used to affinity purify the antibodies.

Using a full-length cDNA clone (AB044534) in pBluescript SK as a template, an ORF of goldfish CPEB was amplified with a primer GFCPEB_ORF_F (Table 1) that introduces a *Bgl*II site just before the first ATG codon and the T7 primer. The resulting PCR product was digested with *Bgl*II and *EcoR*V and ligated into *BamH*I-*EcoR*V-cut pET3a vector (Novagen). Monoclonal antibodies were raised against *E. coli*-produced recombinant proteins.

Digoxigenin (DIG) was coupled with bovine serum albumin 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 obtained from the guinea pig injected with DIG-conjugated bovine serum albumin showed high specificity to DIG, it was used in combination with Alexa 488-labeled secondary antibody to detect DIG-labeled *cyclin B1* antisense probes under a fluorescent microscope for section *in situ* hybridization analysis, as described below.

Immunoblotting and immunoprecipitation (IP)

Immunoblotting was performed as previously described, using anti-IMP3, anti-Pum1 (Pum2A5) (Nakahata et al., 2001), anti-CPEB (GFCPEB 3D3), anti-Cdc2 (MC2-21) (Tanaka and Yamashita, 1995), anti-phospho-MAPK (#9101; Cell Signaling Technology), anti-γ-Tubulin (T6557; Sigma-Aldrich), anti-Cyclin B1 (B112; Katsu et al., 1993), anti-α-Tubulin (T9026; Sigma-Aldrich) or anti-Flag (F1804; Sigma-Aldrich) antibodies.

To examine the interaction of IMP3 with Pum1 or CPEB, 30 μl of oocyte extracts were immunoprecipitated with anti-IMP3, anti-Pum1 or anti-CPEB antibody in the presence of Protein A Sepharose 4 Fast Flow (GE Healthcare) for overnight at 4°C. To examine the dependence of the interaction on mRNA, 5 μl of 10 mg/ml RNase A (Nacalai) was added to the oocyte extracts prior to IP, and the mixture was incubated for 15 min at 37°C. After washing three times in EB, the immunoprecipitates were immunoblotted with anti-IMP3, anti-Pum1, and anti-CPEB antibody.

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

To detect expression of Cyclin B1 protein, crude extract from 15 oocytes were subjected to suc1 precipitation as described previously (Yamashita et al., 1991). Signal intensity was quantified by Image J (NIH).

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 (Kondo et al., 2001). The fractions were also analyzed by immunoblotting to detect Cdc2, Pum1, CPEB and IMP3 and by SDS-PAGE followed by silver staining to detect total protein.

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 IP with anti-Flag antibody and Protein G Sepharose 4 Fast Flow (GE healthcare). The 3' UTRs of zebrafish *cyclin B1* and *β-globin* mRNAs were amplified by PCR with primer sets specific for *cyclin B1* and *β-globin* mRNA 3' UTR sequences (Table 1) and cloned into pGEM T-easy vector. Using the resulting plasmids as templates, DIG-labeled RNA probes were synthesized with T7 RNA polymerase and a DIG RNA labeling mix (Roche). Crosslinking and immunoblotting were performed as described previously (Kotani et al., 2013).

RT-PCR analysis following IP (IP/RT-PCR)

Extracts from 50 oocytes were incubated with anti-IMP3 antibody or anti-Pum1 antibody or without an antibody for 1 h at 4°C. Following the addition of Protein A Sepharose 4 Fast Flow, the extracts were rotated for 3 h at 4°C and washed five times with EB containing 1% Tween 20. After extraction of RNA from the beads 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 primer sets for *cyclin B1*, *mos*, *wee1* and *β-actin* (Table 1).

Section *in situ* hybridization and immunostaining

For toluidine blue staining, 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.

Section *in situ* hybridization of *cyclin B1* mRNA was performed according to the procedure reported previously (Kondo et al., 2001).

For double fluorescent *in situ* hybridization and immunostaining, ovaries were fixed with 4% paraformaldehyde in phosphate buffered saline (4% PFA/PBS) overnight at 4°C, embedded in paraffin, and cut into 12-μm-thick sections. Following deparaffinization and rehydration, sections were hybridized with DIG-labeled antisense RNA probes (Kondo et al., 2001). After washing with TNT buffer (0.1M Tris-HCl, 0.15M NaCl, 0.05% Tween-20, pH7.5), samples were blocked with TNB blocking buffer (0.1M Tris-HCl, 0.15M NaCl, 0.5% (w/v) blocking reagent (PerkinElmer), pH7.5) for 30 min at room temperature and incubated with a mixture of anti-DIG-POD Fab fragments (1:500 dilution; Roche) and anti-IMP3 antibody (1:200 dilution) or anti-Flag antibody (1:500 dilution) diluted in Can Get Signal Immunostain (TOYOBO) for 3 hr at room temperature. After washing, tyramide signal amplification (TSA) was performed with a TSA Plus Fluorescein System (PerkinElmer) according to the manufacturer's instructions. Anti-Flag or anti-IMP3 antibodies were detected with secondary antibodies conjugated with Alexa 546 (Molecular Probes). The sections were observed at room temperature on an LSM 5 LIVE microscope equipped with Plan-Apochromat 10x/0.45 M27 and Plan-Apochromat 20x/0.8 M27 objective lenses (Carl Zeiss). Images were acquired with LSM 5 DUO 4.2 software (Carl Zeiss) and processed with Photoshop and Illustrator (Adobe).

Oocyte microinjection

Manually isolated full-grown oocytes were injected with 2.3 nl of PBS containing 1 μg/μl of mRNAs encoding *Flag-IMP3* or *Flag-GST* using a Drummond Nanoject II microinjector. The same volume of PBS was also injected as a vehicle control. Injected oocytes were transferred to culture medium for 2 hr at RT. After incubation, oocytes were subjected to section *in situ* hybridization and immunostaining. To examine the effects of overexpression on *cyclin B1* mRNA translation, oocytes were transferred to the culture medium containing MIH, harvested after 90 min and lysed for Suc1 precipitation and immunoblotting. To assess the effects on progression of oocyte maturation, the rate of GVBD was monitored at appropriate intervals.

Results

Identification of novel *cyclin B1* mRNA-binding proteins

To isolate proteins bound to *cyclin B1* mRNA, I 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* (Tanaka and Yamashita, 1995), 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. A *cyclin B1* mRNA-binding protein Pum1 (Kotani et al., 2013) was 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. The following five proteins were identified as candidate proteins bound to *cyclin B1* mRNA: IMP3, LSM14 homolog A, DDX6, ELAV-like protein and Zygote arrest protein 1.

Next, I examined whether the candidate proteins can bind to the 3' UTR of *cyclin B1* mRNA by UV cross-linking assay. Although GST as a control did not interact, IMP3 (Fig. 1B) and ELAV-like protein (data not shown) interacted with the *cyclin B1* 3' UTR but not with the control *β-globin* 3' UTR. Unfortunately, I was unable to obtain clear results showing interaction of *cyclin B1* mRNA with the three other candidate proteins. Our previous study identified IMP3 as a component of *cyclin B1* mRNA aggregation isolated from immature zebrafish oocytes by laser capture microdissection (Ota et al., unpublished), I thus decided to focus on IMP3 in this study.

Detection of IMP3 in oocytes

To detect zebrafish IMP3 protein, I produced a mouse polyclonal antibody against a truncated IMP3 (amino acids 197-409), because a full-length version was not expressed in *E. coli*. The antibody recognized a 69-kDa protein in both immature and mature oocytes

(Fig. 2A), in accordance with the molecular mass of *Xenopus* IMP3 (69 kDa) and that estimated from the cDNA sequence of zebrafish IMP3 (ca. 64 kDa). To detect zebrafish CPEB protein, I checked the specificity of monoclonal antibodies which were produced against goldfish CPEB and found that an anti-CPEB antibody (GFCPEB 3D3) specifically recognizes zebrafish CEPB (Fig. 2B). The anti-IMP3-positive 69-kDa protein and the anti-CPEB-positive 60-kDa protein were enriched in heavier fractions after sucrose density gradient ultracentrifugation, similar to *cyclin B1* mRNA and Pum1 (Fig. 1A), confirming that the antibodies recognize IMP3 and CPEB. The protein levels of IMP3 remained constant during oocyte maturation, like γ-Tubulin as a loading control (Fig. 2C). As in the case of *Xenopus* (Ota et al., 2011a), Pum1, CPEB and MAP kinase were phosphorylated during maturation and CPEB was degraded in mature oocytes. Since *Xenopus* IMP3 is phosphorylated during oocyte maturation (Git et al., 2009), I examined incorporation of ^{32}P into zebrafish IMP3 during oocyte maturation by injecting [^{32}P]ATP into oocytes or incubating oocyte extracts with [^{32}P]ATP. However, IMP3 was not labeled with ^{32}P (data not shown). I then examined the phosphorylation state of zebrafish IMP3 by Phos-tag SDS-PAGE (Fig. 2D). Retardation in electrophoretic mobility of zebrafish IMP3 was observed in both immature and mature oocytes, and the retarded bands disappeared with λ-phosphatase treatment. These findings suggest that zebrafish IMP3 is already phosphorylated in immature oocytes and the phosphorylation level remains constant during oocyte maturation.

Interaction between endogenous IMP3 and *cyclin B1* mRNA

Interaction between IMP3 and *cyclin B1* mRNA *in vivo* was investigated by IP/RT-PCR (Fig. 3A). 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 oocyte

maturation like *cyclin B1* mRNA. In contrast, anti-Pum1 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 (Ota et al., 2011a). 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 the mRNA in mature oocytes, while Pum1 remains associated with the mRNA throughout oocyte maturation.

Interaction between IMP3 and Pum1 or CPEB

I examined the interaction between IMP3 and Pum1 by co-IP assay (Fig. 3B). When immature oocyte extracts were used, anti-IMP3 immunoprecipitates contained Pum1 and, conversely, anti-Pum1 immunoprecipitates contained IMP3. However, interaction between IMP3 and Pum1 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 Pum1 remains associated with *cyclin B1* mRNA in mature oocytes (Fig. 2A). In addition, the interaction between Pum1 and IMP3 was disrupted by RNaseA treatment prior to IP. These results revealed that the interaction of IMP3 and Pum1 in immature oocytes is indirect and dependent on the presence of *cyclin B1* mRNA.

In *Xenopus*, Pum1 and CPEB form a complex directly via protein-protein interaction (Nakahata et al., 2003; Ota et al., 2011a). I then investigated the interaction between CPEB and IMP3 by co-IP assay (Fig. 3C). Since the position of CPEB on SDS-PAGE of anti-IMP3 immunoprecipitates overlapped with that of immunoglobulin heavy chains used for IP, it was difficult to detect CPEB in anti-IMP3 immunoprecipitates. I 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 with Pum1.

Subcellular localization of IMP3 in oocytes

Since *cyclin B1* mRNA localizes to the animal polar cytoplasm beneath the micropyle in immature oocytes (Fig. 4A and B), I expected that IMP3 also localizes to the animal polar cytoplasm with *cyclin B1* mRNA. To confirm this, I performed *in situ* hybridization of *cyclin B1* mRNA and immunostaining of IMP3, using the same histological sections (Fig. 4C). The double fluorescent staining demonstrated that IMP3 is distributed throughout the cytoplasm like *cyclin B1* mRNA in young oocytes, and in the later stages, IMP3 is co-localized to the animal polar cytoplasm with *cyclin B1* mRNA. Consistent with the previous observations (Kotani et al., 2013), higher magnification images of the animal polar region in stage IV oocytes showed that *cyclin B1* mRNA forms granules, whereas IMP3 does not (Fig. 4D). In mature oocytes, IMP3 did not exhibit any subcellular localization (data not shown), in harmony with the dispersion of *cyclin B1* mRNA in the cytoplasm (Kondo et al., 2001).

Effects of IMP3 overexpression on translation of *cyclin B1* mRNA and oocyte maturation

To gain an insight into the function of IMP3, I overexpressed Flag-tagged IMP3 in full-grown oocytes and analyzed by immunoblotting and immunostaining (Fig. 5A). Oocytes were microinjected with *Flag-IMP3* mRNA, *Flag-GST* mRNA or PBS and incubated to allow protein expression. Immunoblotting of injected oocytes with anti-Flag antibody showed protein expression from the injected mRNAs (Fig. 5B). It is noteworthy that the overexpressed Flag-IMP3 was localized to the animal polar cytoplasm with endogenous *cyclin B1* mRNA (Fig. 5C), but Flag-GST did not show any localization (data not shown).

The relative amount of Cyclin B1 protein in oocytes overexpressing Flag-IMP3 was significantly lower than those in control oocytes injected with *Flag-GST* mRNA or PBS

(Fig. 6A and B), suggesting that the overaccumulation of IMP3 at the animal polar cytoplasm (Fig. 5C) inhibited the translational activation of *cyclin B1* mRNA. Moreover, oocytes overexpressing IMP3 underwent GVBD at a significantly later timing than did control oocytes (Fig. 6C and D). Since MAP kinase is phosphorylated in response to hormonal stimulation (Fig. 2C), I examined the phosphorylation states of MAP kinase in the injected oocytes, but there was no significant difference (Fig. 6E and F), implying that the effect of overexpression of IMP3 is restricted to certain reactions including *cyclin B1* mRNA translation. Taken together, overexpressed IMP3 repressed translation of *cyclin B1* mRNA and retarded the progression of oocyte maturation.

Discussion

To further understand the mechanisms underlying the translational regulation of *cyclin B1* mRNA, I identified IMP3 as a novel *cyclin B1* mRNA-binding protein in zebrafish oocytes. In immature oocytes, IMP3 specifically associates with *cyclin B1* mRNA, but in mature oocytes, it dissociates from the mRNA. Particularly, overexpression of IMP3 inhibited translational activation of *cyclin B1* mRNA and caused a delay in the timing of oocyte maturation. Although all of the data obtained in this study suggest that IMP3 represses the translation of *cyclin B1* mRNA in immature oocytes, its functional significance remains unclear.

Binding of IMP3 to *cyclin B1* mRNA and their localization to the animal pole

IMP3 was described as a protein bound to the VLE in the 3'UTR of *vg1* mRNA that localizes to the vegetal cortex of *Xenopus* oocytes (thereby also called *vg1* mRNA-binding protein) (Mowry and Melton, 1992; Schwartz et al., 1992). Since the 3' UTR of zebrafish *cyclin B1* mRNA harbors an E2 motif (UUCAC), a *cis*-acting element in the VLE (Deshler et al., 1998; Kwon et al., 2002), 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 (Bally-Cuif et al., 1998), regardless of the lack of an E2 motif in zebrafish *vg1* mRNA. In addition, zebrafish *nanos1* mRNA does not localize to the animal pole (but localizes to the vegetal pole), 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; otherwise, E2 motif is not a *cis*-acting element for IMP3 in

zebrafish oocytes. Further studies are required to understand a general mechanism that localizes different mRNAs to the animal polar cytoplasm in zebrafish oocytes.

Phosphorylation of IMP3

IMP3 is phosphorylated by Erk2/MAP kinase on S402 that exists in the linker separating the second and third KH domains, resulting in the cortical release of *vg1* mRNA during *Xenopus* oocyte maturation (Git et al., 2009). However, S402 is only conserved in *X. laevis* and *Xenopus tropicalis* IMP3 and is absent in other vertebrate homologs including zebrafish IMP3 (Fig. 2E). Consistent with this, I found that zebrafish IMP3 is not newly phosphorylated, despite the fact that MAP kinase is activated after hormonal stimulation (Fig. 2D). 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 remains unchanged during oocyte maturation (Fig. 2D). Irrespective of the steady phosphorylation state, IMP3 dissociates from *cyclin B1* mRNA in mature oocytes (Fig. 3A). 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.

Functions of IMP3 during oocyte growth and maturation

The vertebrate homologs of IMP3 exist in various cell types and play important roles in post-transcriptional regulation such as mRNA transport (Deshler et al., 1998), translational repression (Nielsen et al., 1999), translational activation (Liao et al., 2005), and mRNA stabilization (Vikesaa et al., 2006). In immature *Xenopus* oocytes, IMP3 is bound to *vg1* mRNA that localizes to the vegetal cortex (Mowry and Melton, 1992; Schwartz et al., 1992), and its phosphorylation by MAP kinase during oocyte maturation

induces cortical detachment and solubilization of *vg1* mRNA (Git et al., 2009). Although these findings suggest that IMP3 plays a role in the localization of *vg1* mRNA, it is uncertain whether IMP3 is involved in the translational repression or activation of *vg1* mRNA in *Xenopus* oocytes. In fact, the translation of *vg1* mRNA has been reported to be repressed by Elr-type Proteins in growing oocytes until stage IV but activated thereafter even in the presence of IMP3 (Colegrove-Otero et al., 2005; Arthur et al., 2009). To gain an insight into the function of IMP3 in zebrafish oocytes, I overexpressed IMP3 in oocytes to enhance its function. Overexpression of IMP3 retarded the translation of *cyclin B1* mRNA and the progression of oocyte maturation (Fig. 6A-D), 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 (data not shown). 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 injection experiments suggest that IMP3 represses the translation of *cyclin B1* mRNA in immature oocytes, we need to clarify its actual function. Recently, Yasuda et al. have established an *in vivo* reporter gene assay system in zebrafish, in which the reporter mRNA can precisely mimic the behavior of endogenous *cyclin B1* mRNA and can visualize its timing of translational activation during oocyte maturation in real time. 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.

Chapter II

Staufen1 functions in *cyclin B1* mRNA transport to the animal polar cytoplasm of zebrafish oocytes in collaboration with Kinesin1 and microtubules

Abstract

Precise localization of maternal mRNAs in oocytes is critical for oocyte maturation and embryonic development. In full-grown zebrafish oocytes, dormant *cyclin B1* mRNAs are localized to the animal polar cytoplasm and translationally activated during oocyte maturation. To elucidate the molecular basis of *cyclin B1* mRNA localization, I analyzed zebrafish Staufen1, a protein known to play a central role in mRNA localization in various cells. Zebrafish Staufen1 interacts with *cyclin B1* mRNA throughout oocyte growth and maturation. Both *cyclin B1* mRNA and Staufen1 protein are evenly distributed in the cytoplasm of young oocytes but are co-localized to the animal polar cytoplasm in later stages. In mature oocytes, *cyclin B1* mRNA is dispersed throughout the cytoplasm, while a subpopulation of Staufen1 remains at the animal pole. Transport of *cyclin B1* reporter mRNA to the animal pole was inhibited by disruption of microtubules and injection of antibodies against Staufen1 or Kinesin1, a plus-end-directed microtubule motor that binds to Staufen1, indicating that the transport depends on movement along microtubules toward the plus ends. The plus ends of microtubules are enriched in the animal polar cytoplasm of zebrafish oocytes, contrary to *Xenopus* oocytes, in which they are enriched in the vegetal polar cytoplasm. Reporter mRNAs with an element required for the vegetal localization of *vg1* mRNA in *Xenopus* oocytes were localized to the animal polar cytoplasm in zebrafish oocytes. These findings indicate that a common mRNA transport machinery functions in zebrafish and *Xenopus* oocytes but that its transport direction is opposite probably due to different organizations of microtubules in oocytes.

Introduction

Specific subcellular localization of mRNAs enables spatio-temporal control of gene expression, which assures normal progression of various biological events including embryonic development accompanied by cell proliferation and differentiation (Martin and Ephrussi, 2009; Kumano, 2012; Blower, 2013). For example, localization of *oskar* mRNA to the posterior pole of *Drosophila* oocytes is required to determine the germ cell lineage (Ephrussi and Lehmann, 1992), and *wnt11* mRNA localized at the vegetal cortex of *Xenopus* oocytes is required to determine the body axis (Ku and Melton, 1993; Tao et al., 2005). Specific localization of several mRNAs has also been reported in zebrafish oocytes (Howley and Ho, 2000). One prominent example is *buc* mRNA, the localization of which is indispensable for the establishment of oocyte polarity and assembly of germ plasm (Dosch et al., 2004; Marlow and Mullins, 2008; Bontems et al., 2009; Heim et al., 2014).

Cyclin B1 is a regulatory subunit of MPF, the final inducer of oocyte maturation (Nagahama and Yamashita, 2008). In zebrafish oocytes, Cyclin B1 protein is absent because its mRNA is translationally repressed and localized as aggregates to the animal polar cytoplasm until the oocytes are stimulated by MIH (Kondo et al., 1997, 2001). Cytochalasin B, an inhibitor of actin polymerization, disrupts the localization and translationally activates *cyclin B1* mRNA without MIH stimulation (Kondo et al., 2001). During oocyte maturation, the meshwork of microfilaments beneath the animal pole is reconstructed in response to MIH stimulation, an event probably required to release *cyclin B1* mRNA from translational repression at the oocyte cortex (Nukada et al., 2015). The results of these studies indicate that microfilaments play a key role in the localization and translational repression of *cyclin B1* mRNA in zebrafish oocytes.

By using an experimental system in which reporter mRNAs can precisely mimic the behavior of endogenous *cyclin B1* mRNAs in zebrafish oocytes, the previous studies revealed that the translational timing of *cyclin B1* mRNA after MIH stimulation is regulated

by at least two components, a *cis*-acting element situated in the ORF and Pum1 bound to the PBE in the 3' UTR of *cyclin B1* mRNA (Yasuda et al., 2010, 2013; Kotani et al., 2013). Notable findings obtained in these experiments are that *cyclin B1* reporter mRNA with mutations in the ORF element and PBE failed to form aggregation and underwent precocious translational activation after MIH stimulation, but the transport of mRNAs to the animal polar cytoplasm was not affected (Yasuda et al., 2010, 2013). These results indicate that *cyclin B1* mRNA transport to the animal polar cytoplasm is independent of the ORF element, Pum1 and microfilaments, all of which are necessary for translational activation of the mRNA at appropriate timing during oocyte maturation. The aim of this study was to determine the transport mechanism of *cyclin B1* mRNA in zebrafish oocytes.

I identified IMP3 as a *cyclin B1* mRNA-binding protein and suggested its involvement in translational regulation in Chapter I. IMP3 is a member of the VICKZ family, which is involved in various post-transcriptional regulations such as mRNA transport and translational regulation (Yisraeli, 2005). Especially in *Xenopus* oocytes, IMP3 (also known as Vg1RBP) plays a crucial role in the localization of *vg1* mRNA to the vegetal cortex (the localization machinery being called the late (vg1) pathway: Deshler et al., 1998, 1997; Elisha et al., 1995; Git et al., 2009; Schwartz et al., 1992). Interestingly, IMP3 and its binding targets, *vg1* mRNA and *cyclin B1* mRNA, are localized to the animal polar cytoplasm in zebrafish oocytes (Bally-Cuif et al., 1998), implying that a pathway similar to the late pathway in *Xenopus* oocytes exists for the animal polar localization of *vg1* and *cyclin B1* mRNAs in zebrafish oocytes. Stau1, a double-stranded RNA-binding protein, is thought to play an important role in the late pathway of *Xenopus* oocytes (Allison et al., 2004; Yoon and Mowry, 2004). Originally, Stau1 was identified in a *Drosophila* mutant in which localization of maternal mRNAs in oocytes was abrogated (St Johnston et al., 1989, 1991). Stau1 has been shown to regulate various post-transcriptional events including translational activation (Dugré-Brisson et al., 2005), stress granule assembly (Thomas et al.,

2009), alternative splicing (Ravel-Chapuis et al., 2012; Bondy-Chorney et al., 2016) and mRNA degradation called Staufen-mediated mRNA decay (Park and Maquat, 2013). Stau1 is also involved in cell cycle progression in cancer cells (Boulay et al., 2014), embryonic stem cell differentiation (Gautrey et al., 2008) and life cycles of various RNA viruses as a host protein (Abrahamyan et al., 2010; de Lucas et al., 2010; Blackham and McGarvey, 2013). In zebrafish, *stau1* mRNA is highly expressed in the brain and gonads (Bateman et al., 2004), and its inhibition by antisense morpholino oligonucleotides results in aberrant survival and migration of primordial germ cells (Ramasamy et al., 2006). Taken together, these facts indicate the possibility that Stau1 is involved in *cyclin B1* mRNA transport to the animal polar cytoplasm in zebrafish oocytes, and I thus attempted to investigate this possibility in this study.

I examined zebrafish Stau1 from oocyte growth through to maturation and found that Stau1 is bound to *cyclin B1* mRNA and is localized to the animal polar cytoplasm. I also found that *cyclin B1* mRNA transport to the animal pole is inhibited by a microtubule inhibitor, nocodazole, and antibodies against Stau1 and Kinesin1. These findings suggest the function of Stau1 in *cyclin B1* mRNA transport to the animal polar cytoplasm in collaboration with Kinesin1 and microtubules. Furthermore, I discovered that the plus ends of microtubules are enriched in the animal polar cytoplasm of full-grown oocytes, suggesting that *cyclin B1* mRNA-Stau1 protein complexes are transported toward the animal pole by the plus-end-directed motor protein Kinesin1 along microtubules. Based on these findings, I propose that the transport machinery of *cyclin B1* mRNA in zebrafish oocytes is compositionally similar to that of *vg1* mRNA in *Xenopus* oocytes, although. the transport direction is different.

Materials and Methods

Oocyte culture and extraction

Protein extraction from oocytes were performed as described in Chapter I. Total protein concentration of the extracts were quantified by the Bradford assay using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) following the manufacturer's instructions. For RNA analysis, RNasin Plus RNase inhibitor (100 units/ml at the final concentration, Promega) was added to the extracts. For IP analysis, Triton X-100 (1% at the final concentration) was added to the extracts.

Production of recombinant proteins and antibodies

To produce N-terminally Flag-tagged Stau1 (Flag-Stau1), an ORF of zebrafish Stau1 was amplified with a primer set of Stau1_F and Stau1_R (Table 2). The PCR product was inserted into the *EcoR*I-digested pCS2+FT-N vector (Ota et al., 2011b) using an In-Fusion HD Cloning Kit (TaKaRa). To produce *Flag-stau1* mRNA, the sequence between SP6 and T3 promoter of the resulting plasmid was amplified by PCR and used for *in vitro* transcription with an mMESSAGE mMACHINE SP6 kit (Ambion).

A cDNA fragment of zebrafish Stau1 was amplified with a primer set of Stau1_exF and Stau1_exR (Table 2) and inserted into the pENTR/D-TOPO Gateway vector (Invitrogen). The resulting plasmids were recombined with the destination vector pET161-DEST using a Gateway cloning system (Invitrogen) to produce Stau1 tagged with a polyhistidine (His) at the C terminus (Stau1-His). Stau1-His was expressed in *E. coli* BL21 (DE3) and purified according to the method described previously (Ota et al., 2008). Polyclonal antibodies against Stau1-His were produced in mice (anti-Stau1 (M)) and guinea pigs (anti-Stau1 (GP)) and affinity-purified with antigenic Stau1-His protein as described in Chapter I. Similarly, an anti-EB1 mouse antibody was raised against zebrafish EB1 produced with a primer set of EB1_exF and EB1_exR (Table 2).

Immunoblotting and IP

Immunoblotting was performed as described previously (Ota et al., 2011a) using anti-Stau1 (M), anti-Stau1 (GP), anti-EB1, anti-IMP3 (Chapter I), anti-α-Tubulin (T9026; Sigma-Aldrich), anti-Pum1 (Kotani et al., 2013), anti-RPL11 (ab79352; abcam), or anti-Flag (F1804; Sigma-Aldrich) antibodies. Interaction of Stau1 with Pum1 or IMP3 was examined by IP, as described in Chapter I. To detect phosphorylation of Stau1, immature and mature oocyte extracts were immunoprecipitated with anti-Stau1 (GP) antibody, and the resulting precipitates were treated with λ-phosphatase (200 units, New England Biolabs) for 1 hr at 30°C. The samples were separated by SDS-polyacrylamide gel containing 40 μM Phos-tag acrylamide (Wako) and 80 μM $MnCl_2$ and immunoblotted with anti-Stau1 (M) antibody.

RT-PCR and qRT-PCR

Oocyte extracts (100 μg protein) were incubated with anti-Flag or anti-Stau1 (GP) antibody or preimmune serum as a control in the presence of protein G- or A-Sepharose beads (GE Healthcare) for 3 hr at 4°C. After washing six times in EB containing 1% Triton X-100, co-immunoprecipitated RNAs were extracted from the beads by ISOGEN II (Nippon gene). For RT-PCR analysis, cDNA was synthesized with a SuperScript IV First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed with a Taq DNA Polymerase (Ampliqon). For qRT-PCR analysis, cDNA was synthesized with a PrimeScript RT Master Mix (Perfect Real Time) (TaKaRa) according to the manufacturer's instructions. Real-time PCR amplification was performed with a SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa) on Thermal Cycler Dice Real Time System II (TaKaRa). Enrichment of each mRNA in anti-Stau1 (GP) immunoprecipitates relative to that in the control precipitates with preimmune serum was calculated, and the obtained value of each mRNA was statistically analyzed by two-tailed

unpaired Student's *t*-test compared with that of *β-actin*. To quantify reporter mRNAs, total RNA extracted from 10 injected oocytes was subjected to qRT-PCR using primer sets for *gfp* and *β-actin*. The content of *gfp* reporter mRNAs was normalized to that of *β-actin* mRNA. No *gfp* reporter mRNAs were detected when non-injected oocytes were used. The primer sequences used for PCR amplifications are shown in Table 2.

Section *in situ* hybridization, immunostaining and whole-mount *in situ* hybridization

Double fluorescent *in situ* hybridization and immunostaining were performed as described in Chapter I using anti-Stau1 (M) antibody (1:500 dilution) and anti-EB1 antibody (1:200 dilution).

Whole-mount *in situ* hybridization was performed according to the procedure reported previously (Schulte-Merker et al., 1992). In brief, fixed oocytes were treated with 100% methanol and stored at -20°C until further treatment. The oocytes were rehydrated and treated with 10 μg/ml proteinase K in PBS containing 0.1% Tween-20 for 20 min at 37°C with gentle shaking. After hybridization with a DIG-labeled antisense GFP probe and washing, the oocytes were incubated with anti-DIG-POD Fab fragments (1:1,000 dilution; Roche) and developed with diaminobenzidine.

Oocyte microinjection

To overexpress Flag-tagged Stau1, manually isolated full-grown oocytes were injected with 2.3 nl of distilled water containing 2.3 ng *Flag-Stau1* mRNA into the nucleus using a Drummond Nanoject II microinjector. Following incubation in culture medium for 2 hr at room temperature to allow protein expression, 100 oocytes were lysed and subjected to anti-Flag IP and RT-PCR analysis.

A VLE reporter construct was produced by replacing the *cyclin B1* 3'UTR of *tgo3'* (Yasuda et al., 2010) with the *Xenopus* VLE sequence. Using total cDNA from *Xenopus* oocytes as a template, *Xenopus* VLE was amplified with a primer set of xVg1VLE_F and xVg1VLE_R (Table 2) and cloned into the *tgo3'* vector amplified with a primer set of cyclinB1_ORF_R and pT2KXIGΔin_BglII_F (Table 2) using an In-Fusion HD Cloning Kit (TaKaRa). The SV40 reporter construct was produced as described previously (Yasuda et al., 2013).

For an *in vivo* reporter mRNA localization assay, oocytes were injected with distilled water containing 250 pg of reporter vectors and either 10 ng/μl of nocodazole, 100 ng/μl of anti-GST (Ota et al., 2011a), anti-Kinesin1 (SUK4; DSHB) or anti-Stau1 (M) antibodies. After injection, oocytes were incubated in culture medium for 4 hr at room temperature. The oocytes were fixed with 4% PFA/PBS overnight at 4°C and subjected to whole-mount *in situ* hybridization. Localized signals were classified into five groups and counted according to a previous study (Yasuda et al., 2010).

Results

Behavior of zebrafish Stau1 during oocyte growth and maturation

To detect zebrafish Stau1, I produced antibodies against the C-terminal half of Stau1 in the mouse (M) and guinea pig (GP). Both antibodies recognized a single band of ca. 75 kDa, the size of which is comparable to that of Stau1 consisting of 702 amino acids, in oocyte extracts and immunoprecipitates (Fig. 7A, B). Since Flag-tagged Stau1 expressed in rabbit reticulocyte lysate also showed a similar size (data not shown), I conclude that the 75-kDa band is Stau1 in zebrafish oocytes. In this study, anti-Stau1 (M) antibody was used in immunoblotting, immunostaining and microinjection, and anti-Stau1 (GP) antibody was used in immunoblotting and IP.

The expression levels of Stau1 during oocyte growth and maturation were examined by immunoblotting using oocytes classified into 4 stages (Stages I to III are growing stages and stage IV is the full-grown stage.) (Selman et al., 1993). Stage IV oocytes were induced to mature in order to obtain stage V oocytes. Zebrafish Stau1 was predominantly expressed in oocytes of stages I to III, and its level decreased in stages IV and V (Fig. 7C). I also examined the phosphorylation state of Stau1 in stages IV and V by Phos-tag SDS-PAGE (Fig. 7D). Retardation in electrophoretic mobility of Stau1 was observed only in mature (stage V) oocytes, and the retarded bands disappeared with λ-phosphatase treatment, indicating that Stau1 is phosphorylated during oocyte maturation.

Interaction of Stau1 with *cyclin B1* mRNA in oocytes

I analyzed the interaction of Stau1 with *cyclin B1* mRNA in oocytes by RT-PCR following IP. Full-grown oocytes that overexpress Flag-tagged Stau1 were immunoprecipitated with anti-Flag antibody, and *cyclin B1* mRNA in the precipitates was detected by RT-PCR. The results clearly showed that *cyclin B1* mRNA was present in

anti-Flag immunoprecipitates from Flag-Stau1-overexpressing oocytes but not in control precipitates (Fig. 8A), indicating that Flag-tagged Stau1 binds to *cyclin B1* mRNA in oocytes.

Since Pum1 (Kotani et al., 2013) and IMP3 (Chapter I of this thesis) are *cyclin B1* mRNA-binding proteins in zebrafish oocytes, I examined the interaction between Stau1 and Pum1 (Fig. 8B) or IMP3 (Fig. 8C) in full-grown (stage IV) oocytes by a co-IP assay. Anti-Pum1 immunoprecipitates contained Stau1 and, conversely, anti-Stau1 immunoprecipitates contained Pum1. Similarly, anti-IMP3 immunoprecipitates contained Stau1, and anti-Stau1 immunoprecipitates contained IMP3. These results were canceled by RNase A treatment prior to IP, indicating that Stau1 does not bind directly to Pum1 or IMP3 but binds via *cyclin B1* mRNA. Since the interaction between Pum1 and IMP3 is also RNA-dependent (Chapter I of this thesis), it is likely that these three proteins independently bind to *cyclin B1* mRNA.

Interaction of Stau1 with *cyclin B1* mRNA during oocyte growth and maturation (stages I to V) was analyzed by IP followed by qRT-PCR (Fig. 8D). In all of the stages, *cyclin B1* mRNA contents in anti-Stau1 immunoprecipitates were 7- to 11-fold higher than that in control immunoprecipitates using preimmune serum. Moreover, they were significantly higher than the content of *β-actin* mRNA (a control mRNA because it is assumed to disperse throughout the oocyte cytoplasm), indicating that Stau1 preferentially binds to *cyclin B1* mRNA. The *cyclin B1* mRNA contents in anti-Stau1 immunoprecipitates did not change at statistically significant levels from stage I through stage V, although they tended to decrease with oocyte growth. These results indicate that Stau1 continues to interact with *cyclin B1* mRNA during oocyte growth and maturation, in striking contrast to the fact that localization of *cyclin B1* mRNA and Stau1 changes during oocyte growth and maturation as described below (cf. Fig. 9).

I also examined the interactions between Stau1 and other mRNAs that have been reported to show characteristic localization patterns in zebrafish oocytes (Fig. 8E; see

Discussion for the characteristic localization patterns of mRNAs examined in this study). I found that *cyclin B1* (a positive control), *zorba* (*cpeb*), *mos*, *vg1* and *buc* mRNAs (their final destination being the animal polar cytoplasm of full-grown oocytes) have already interacted with Stau1 in stage I and II (growing) oocytes, in which *cyclin B1*, *zorba*, *mos* and *vg1* mRNAs are distributed throughout the cytoplasm and *buc* mRNA is localized to the Balbiani body (mitochondrial cloud). In contrast, I could not detect significant interaction between Stau1 and mRNA of *dazl*, *brul*, or *vasa* (their final destination being the vegetal polar cytoplasm or the cortex of full-grown oocytes) in oocytes of all stages examined in this study. In stage IV (full-grown) oocytes, the interactions between Stau1 and *cyclin B1*, *zorba* (*cpeb*), *mos* and *buc* mRNAs were maintained, but the interaction between Stau1 and *vg1* mRNA was below a statistically significant level.

Subcellular distribution of Stau1 and *cyclin B1* mRNA during oocyte growth and maturation

It is known that the subcellular distribution of *cyclin B1* mRNA changes during oocyte growth and maturation in zebrafish (Howley and Ho, 2000; Kondo et al., 2001). Since Stau1 interacts with *cyclin B1* mRNA (Fig. 8D), I investigated the subcellular distribution of Stau1 in oocytes from stages I to V. In stage I oocytes, Stau1 was distributed throughout the cytoplasm like *cyclin B1* mRNA, with strong signals at the cortical cytoplasm of oocytes (Fig. 9A1-3). In stage II oocytes, signals of both *cyclin B1* mRNA and Stau1 became weaker than those in stage I oocytes, and some of them were localized to the animal polar cytoplasm (Fig. 9B1-3). In stage III oocytes, the vast majority of *cyclin B1* mRNA was localized to the animal polar cytoplasm. Although the majority of Stau1 was also localized to the animal polar cytoplasm in this stage, a subpopulation of Stau1 was localized to the cortical cytoplasm other than the animal polar region and the periphery of the nucleus (Fig. 9C1-6). In stage IV oocytes (full-grown immature oocytes), *cyclin B1* mRNA and Stau1

were tightly co-localized to the animal polar cytoplasm. Stau1 was also detected in the cortical cytoplasm and the periphery of the nucleus (Fig. 9D1-6) as in the previous stage. In stage V oocytes (mature oocytes), the signal of *cyclin B1* mRNA disappeared in accordance with its dispersion into the whole cytoplasm (Kondo et al., 2001; Kotani et al., 2013). In striking contrast to this, the signal of Stau1 remained in the animal polar cytoplasm and the cortical cytoplasm of mature oocytes (Fig. 9E1-6). To resolve this discrepancy, I observed *cyclin B1* mRNA and Stau1 during oocyte maturation and found that *cyclin B1* mRNA and Stau1 were synchronously dispersed from the animal polar cytoplasm after hormonal stimulation (Fig. 9F and G), indicating that only a subpopulation of Stau1 remains in the animal polar cytoplasm after oocyte maturation.

Involvement of microtubules, Kinesin1 and Stau1 in *cyclin B1* mRNA transport to the animal pole

A newly developed experimental system can precisely mimic the behavior of endogenous *cyclin B1* mRNAs in zebrafish oocytes (Yasuda et al., 2010, 2013). In this system, a DNA construct that expresses *gfp-cyclin B1* reporter mRNA under the control of the *Xenopus* EF1α promoter (Fig. 10A) was injected into the nucleus of stage IV oocytes. Whole-mount *in situ* hybridization analysis showed that reporter mRNAs are detected as aggregated signals in the animal polar cytoplasm (cf. Fig. 12B), as in the case of endogenous *cyclin B1* mRNA. In addition, anti-Stau1 IP analysis confirmed interaction between Stau1 and reporter mRNA in oocytes (Fig. 10B). I therefore used this system to examine the role of Stau1 in transport and localization of *cyclin B1* mRNA.

I investigated the behaviors of *cyclin B1* reporter mRNAs under various conditions. First, the requirement of microtubules for *cyclin B1* mRNA transport was examined by injection of 10 ng/μl nocodazole into oocytes, since Stau1 is known to function in microtubule-dependent mRNA transport in many cells including *Drosophila* oocytes

(Ferrandon et al., 1994) and mammalian neurons (Kiebler et al., 1999; Köhrmann et al., 1999). Injection of nocodazole abolished the aggregation of reporter mRNAs at the animal pole, indicating that the transport of reporter mRNA depends on microtubules (Fig. 10C).

Next, I examined the cooperation between Stau1 and Kinesin1 (a motor protein that moves toward the plus ends of microtubules) in reporter mRNA transport, because several lines of evidence suggest interaction between Stau1 and Kinesin1 (Brendza et al., 2000; Yoon and Mowry, 2004). In contrast to injection of water or anti-GST antibody as controls, injection of anti-Kinesin1 or anti-Stau1 antibodies decreased the percentage of oocytes that showed aggregation of reporter mRNAs (Fig. 10C), despite the fact that real-time PCR analysis showed no statistically significant differences in expression levels of reporter mRNAs under various conditions (Fig. 10D). These results suggest that microtubules, Kinesin1 and Stau1 have important roles in *cyclin B1* mRNA transport to the animal pole in zebrafish oocytes.

Microtubule orientation in zebrafish oocytes.

My results suggested that Kinesin1 is a key molecule for *cyclin B1* mRNA transport toward the animal polar cytoplasm in zebrafish oocytes. Since Kinesin1 is a motor protein that drives cargos toward the plus ends of microtubules, I investigated the orientation of microtubules in zebrafish oocytes by immunostaining of EB1, a microtubule plus-end tracking protein (Mimori-Kiyosue et al., 2000).

A newly produced antibody that recognizes zebrafish EB1 (Fig. 11A and B) revealed that EB1 accumulated in the animal polar cytoplasm of full-grown oocytes and co-localized with *cyclin B1* mRNA in part (Fig. 11C and D), whereas it showed no clear accumulation in other regions including the vegetal polar cytoplasm (Fig. 11E). It is therefore likely that the majority of microtubules tend to direct their plus ends toward the animal pole in full-grown zebrafish oocytes.

VLE-directed mRNA transport in zebrafish oocytes.

In *Xenopus* oocytes, Stau1 transports *vg1* mRNA to the vegetal polar cytoplasm via a *cis*-acting element of the mRNA called VLE (Mowry and Melton, 1992; Yoon and Mowry, 2004). If VLE is also functional in zebrafish oocytes, what effect does VLE exert on mRNA localization? To answer this question, I analyzed the behavior of *cyclin B1* reporter mRNA harboring VLE instead of *cyclin B1* 3' UTR (Fig. 12A, VLE) (Yasuda et al., 2013). Consistent with results previously reported (Kotani et al., 2013; Yasuda et al., 2013), *cyclin B1* reporter mRNAs harboring SV40 polyadenylation signal (a negative control) (Fig. 12A, SV40) were not localized to any regions (Fig. 12B). When *cyclin B1* reporter mRNA with the 3' UTR (a positive control) was expressed in oocytes, definite aggregated signals were detected in about 45% of the oocytes. In contrast, about 12% of the oocytes showed definite aggregated signals when reporter mRNA harboring VLE was expressed. However, the ratio of oocytes with aggregated signals reached about 70% in the positive control and 60% in the VLE experiment when class I oocytes (refer to Fig. 12B) were counted as those exhibiting aggregated signals (Fig. 12C). It is therefore likely that the VLE sequence is functional in zebrafish oocytes as a *cis*-acting element for animal polar localization, different from its original function as an element for vegetal polar localization in *Xenopus* oocytes.

How is VLE involved in animal polar localization? One possible explanation is that VLE reporter mRNA is bound to Stau1 and transported to the animal pole. To verify this hypothesis, I checked interaction between Stau1 and reporter mRNAs by anti-Stau1 IP followed by RT-PCR. SV40 reporter mRNA was not detected in anti-Stau1 immunoprecipitates, whereas both VLE and 3' UTR reporter mRNAs were detected (Fig. 12D), indicating that Stau1 binds to VLE reporter mRNA. Although I could not examine direct interaction between Stau1 and VLE or *cyclin B1* 3' UTR, it is plausible that VLE is

recognized by Stau1, which is a component of the mRNA transport machinery to the animal pole of zebrafish oocytes.

Discussion

Animal polar localization of *cyclin B1* mRNA in zebrafish oocytes is critical for oocyte maturation. In this study, I examined the function of Stau1 in *cyclin B1* mRNA transport, since Stau1 is known to participate in various post-transcriptional events including translational regulation. I found that 1) Stau1 is bound to *cyclin B1* mRNA and is localized to the animal polar cytoplasm, 2) *cyclin B1* mRNA transport to the animal pole is inhibited by a microtubule inhibitor and antibodies against Stau1 and Kinesin1, 3) the plus ends of microtubules are enriched in the animal polar cytoplasm in full-grown oocytes, and 4) a reporter mRNA with a *cis*-acting element for Stau1 (VLE) is transported to the animal pole in zebrafish oocytes. Based on these findings, I suggest that *cyclin B1* mRNA is transported to the animal polar cytoplasm toward the plus ends of microtubules by a machinery including Stau1 and Kinesin1 as its components.

Binding targets of Stau1 in zebrafish oocytes

After hormonal stimulation, *cyclin B1* mRNA is translationally activated, which is coincident with dispersion of *cyclin B1* mRNA and Stau1 from the animal polar cytoplasm (Fig. 9F and G). Although *cyclin B1* mRNA was completely detached from the animal pole and eventually became undetectable, a part of Stau1 remained at the cortex (Fig. 9E). Moreover, parts of Stau1 are distributed around the cortical cytoplasm and the nucleus in and after stage III (Fig. 9), inconsistent with *cyclin B1* mRNA localization. Besides *cyclin B1* mRNA, Stau1 is likely to interact with various mRNAs in oocytes.

To identify Stau1 targets other than *cyclin B1* mRNA, I performed anti-Stau1 IP followed by qRT-PCR analysis for several mRNAs that show characteristic localization patterns in zebrafish oocytes (Fig. 8E). The localization patterns of mRNAs examined in this study are as follows: *cyclin B1*, *zorba*, *mos* and *vg1* mRNAs are distributed throughout the

cytoplasm of oocytes from stages I to II and are subsequently localized to the animal polar cytoplasm in full-grown oocytes (Bally-Cuif et al., 1998; Howley and Ho, 2000; Suzuki et al., 2009); *dazl*, *brul*, *vasa* and *buc* mRNAs are localized to the Balbiani body (mitochondrial cloud) in stages I and II, and *dazl* and *brul* mRNAs are then translocated to the vegetal polar cytoplasm (Suzuki et al., 2000; Kosaka et al., 2007), *vasa* mRNA is concentrated around the cortical cytoplasm, and *buc* mRNA is localized to the animal polar cytoplasm (Braat et al., 1999; Howley and Ho, 2000; Bontems et al., 2009). My results demonstrated that Stau1 preferentially binds to mRNAs that are finally localized to the animal polar cytoplasm of full-grown oocytes (Fig. 8E). It is therefore plausible that Stau1 has a general function in mRNA transport to the animal pole in zebrafish oocytes with low selectivity for mRNA species.

Microtubule-mediated *cyclin B1* mRNA transport to the animal polar cytoplasm

To investigate the mechanisms underlying *cyclin B1* mRNA transport to the animal polar cytoplasm, I performed an *in vivo* reporter mRNA localization assay and revealed the involvement of microtubules in the transport (Fig. 10C). On the other hand, Kondo et al. (2001) reported that microfilaments are important for anchoring *cyclin B1* mRNA at the animal polar cytoplasm. The fact that the localization of *cyclin B1* mRNA is established by two processes, mRNA transport via microtubules and mRNA anchoring via microfilaments, is consistent with the two-step model for the localization of *vg1* mRNA to the vegetal cortex in *Xenopus* oocytes (Yisraeli et al., 1990). In addition, it has been reported that microtubule-dependent localization of *vg1* mRNA in *Xenopus* oocytes was inhibited by injection of an anti-Kinesin1 monoclonal antibody (Messitt et al., 2008). Using the same antibody, I confirmed inhibition of *cyclin B1* mRNA localization in zebrafish oocytes (Fig. 10C), indicating that a common microtubule- and Kinesin1-dependent transport mechanism is employed in zebrafish and *Xenopus* oocytes.

In previtellogenic zebrafish oocytes, *nanos* and *dazl* mRNAs, which localize to the vegetal cortex of full-grown oocytes, are present in the Balbiani body situated between the nucleus and the vegetal pole (Kosaka et al., 2007), implying that the animal-vegetal asymmetry has been already established in this stage (stage I). In contrast, *cyclin B1* mRNA and Stau1 are bound to each other (Fig. 8D) but distributed throughout the cytoplasm of stage I oocytes (Fig. 9A). These findings suggest that the binding of Stau1 to *cyclin B1* mRNA is insufficient to initiate mRNA transport. What is required to initiate *cyclin B1* mRNA transport? I found that protein contents of α-Tubulin are low in stage I oocytes and increase with oocyte growth (Fig. 7C), raising the possibility that microtubules are not fully organized to a sufficient level for transporting the Stau1-*cyclin B1* mRNA complex to the animal pole in stage I oocytes. Further studies are required to understand what organization of microtubules is required for *cyclin B1* mRNA transport and what molecules are involved in it.

Presence of a common machinery for *cyclin B1* and *vg1* mRNAs in zebrafish and *Xenopus* oocytes

To gain an insight into the functional conservation of Stau1 in zebrafish and *Xenopus* oocytes, I observed the behavior in zebrafish oocytes of reporter mRNA harboring VLE, a sequence essential for vegetal polar transport in *Xenopus* oocytes. VLE reporter mRNA failed to show definite aggregated signals, but it was transported to the animal polar cytoplasm in zebrafish oocytes (Fig. 12C), presumably depending on the binding of Stau1 to VLE (Fig. 12D). These results suggest that Stau1 has a conserved function in the intracellular transport of mRNAs harboring zebrafish *cyclin B1* 3'UTR and *Xenopus* VLE, although the directions of transport are opposite (animal pole in zebrafish and vegetal pole in *Xenopus*).

In *Xenopus* oocytes, there are two major mRNA localization pathways called early (METRO) pathway and late (vg1) pathway, both of which independently transport their target maternal mRNAs to the vegetal polar cytoplasm (Fig. 13) (Kloc and Etkin, 1995, 2005;

Mowry and Cote, 1999; King et al., 2005). In the early pathway, germ plasm components such as *Xcat2* (*nanos*) mRNA and its *trans*-acting protein Hermes (Rbpms) are transported to the vegetal polar cytoplasm via the mitochondrial cloud (Zhou and King, 1996; Aguero et al., 2016). Based on the finding that a similar pathway via the mitochondrial cloud (also known as the Balbiani body) exists in zebrafish oocytes, Kosaka et al. (2007) suggested that the early pathway is conserved between *Xenopus* and zebrafish oocytes.

In contrast to the early pathway, the late pathway exhibits a species-specific difference between zebrafish and *Xenopus*. Its components such as *vg1* mRNA and its *trans*-acting protein Vg1RBP (IMP3) are localized to the animal polar cytoplasm in zebrafish oocytes, whereas they are localized to the vegetal polar cytoplasm in *Xenopus* oocytes (Bally-Cuif et al., 1998). In this study, I also showed that Stau1, a component of the late pathway, localized to the animal polar cytoplasm in zebrafish (Fig. 9). However, I found that reporter mRNAs with VLE of *Xenopus vg1* mRNA exhibit animal polar localization in zebrafish oocytes (Fig. 12). This finding leads us to propose that *cyclin B1* mRNA transport to the animal polar cytoplasm in zebrafish oocytes is operated by a mechanism conserved in the late pathway of *Xenopus* oocytes, irrespective of the difference in transporting direction. Concerning the difference in direction, it is noteworthy that the microtubule plus end-tracking protein EB1 accumulates in the animal polar cytoplasm of zebrafish oocytes (Fig. 11), contrary to its localization in the vegetal polar cytoplasm of *Xenopus* oocytes (Messitt et al., 2008). Differences in the direction of mRNA transport might be simply explainable by differences in the microtubule organization between zebrafish and *Xenopus* oocytes.

My study has revealed the general and species-specific aspects of mechanisms that regulate maternal mRNA localization in fish and frog oocytes. The difference in the mechanisms should be closely linked to their embryonic development with different cell division patterns, holoblastic versus meroblastic cleavage. Further studies to analyze mRNA transport machineries in oocytes should provide insights into the generality, diversity and

evolution of mechanisms underlying the localization of maternal mRNAs, which will also lead to an understanding of developmental mechanisms, especially for spatio-temporal translational regulation, which is indispensable for cell and tissue differentiation during embryonic organogenesis.

General Discussion

In this study, I investigated the mechanisms regulating the translation and localization of *cyclin B1* mRNA, which play a critical role in the progression of meiotic maturation in zebrafish oocytes. In Chapter I, I characterized IMP3 as a protein involved in the translational repression of *cyclin B1* mRNA, and in Chapter II, I characterized Stau1 as a protein involved in the animal polar localization of *cyclin B1* mRNA. Although the functions of both IMP3 and Stau1 have been reported in various cell types, my study showed their participation in the post-transcriptional regulation of *cyclin B1* mRNA in oocytes for the first time. Hereafter, I discuss the mechanisms of post-transcriptional regulation of *cyclin B1* mRNA in oocytes comprehensively.

Common components involved in translational regulation of *vg1* mRNA in *Xenopus* oocytes and *cyclin B1* mRNA in zebrafish oocytes

The results obtained in this study strongly suggest that a common machinery consisting at least of IMP3 and Stau1 functions for vegetally localized *vg1* mRNA in *Xenopus* oocytes and animally localized *cyclin B1* mRNA in zebrafish oocytes. In Chapter I, I identified ELAV-like protein as a candidate for a *cyclin B1* mRNA-binding protein by using sucrose density gradient ultracentrifugation followed by mass spectrometry analysis. ELAV-like proteins bind to AU-rich elements (AREs) and regulate their stability and/or translation (Peng et al., 1998; Ford et al., 1999). In human colorectal carcinoma RKO cells, one of the ELAV-like family protein HuR binds to and stabilizes *cyclin A* and *B1* mRNAs during cell proliferation (Wang et al., 2000). AREs are found in the 3' UTR of zebrafish *cyclin B1* mRNA, and I checked the interaction of HuR and *cyclin B1* mRNA 3 ’UTR *in vitro* by using UV cross-linking assay (data not shown). In addition, their co-localization at the animal polar cytoplasm of full-grown zebrafish oocytes has been shown (Kotani et al., 2013). Moreover,

the *Xenopus* ELAV-like protein and ARE in the 3' UTR of *vg1* mRNA are responsible for the translational repression of *vg1* mRNA during oogenesis (Otero et al., 2001; Colegrove-Otero et al., 2005). Taken together, it is highly likely that HuR stabilizes and translationally represses *cyclin B1* mRNA in zebrafish oocytes.

Besides IMP3, Stau1 and HuR, many RNA-binding proteins such as hnRNP I (Cote et al., 1999; Kress et al., 2004), Prrp (Zhao et al., 2001), VgRBP71 (Kroll et al., 2002; Kolev and Huber, 2003) and 40LoVe (Czaplinski et al., 2005; Czaplinski and Mattaj, 2006; Kroll et al., 2009) are reported to interact with *vg1* mRNA and to regulate its localization and/or translation in *Xenopus* oocytes. Investigation of the functions of these *Xenopus* proteins in zebrafish oocytes might shed light on the comprehensive understanding of the mechanisms regulating the translation and localization of *cyclin B1* mRNA in zebrafish oocytes.

From transcription to translation of *cyclin B1* mRNA

When *in vitro* transcribed *gfp-cyclin B1* reporter mRNAs were injected into the cytoplasm, the reporter mRNAs were diffused in oocyte cytoplasm and were translationally activated at a timing much earlier than that of endogenous *cyclin B1* mRNA, in distinct contrast to those injected into the nucleus as well as endogenous *cyclin B1* mRNA (Yasuda et al., 2010). This result indicates that the nuclear experience of *cyclin B1* mRNAs is important for the proper regulation. Indeed, several RNA-binding proteins including hnRNP I, Vg1RBP (IMP3) and CPEB interact with their target mRNA in the nucleus and regulate mRNA localization and translation in *Xenopus* oocytes (Kress et al., 2004; Lin et al., 2010). Based on these results, I propose a model for the post-transcriptional regulation of *cyclin B1* mRNA throughout oocyte growth and maturation (Fig. 14).

Through stages I to IV, RNA-binding proteins including IMP3 and CPEB capture *cyclin B1* mRNA in the nucleus and repress its translation upon nuclear export. In young (stage I) oocytes, *cyclin B1* mRNAs exported from the nucleus bind to Stau1 and Pum1 but passively

diffuse in oocyte cytoplasm without any localization, probably because of the absence of microtubules organized to transport mRNAs toward the animal pole (Fig. 14A). During stages II to IV, microtubules assemble into appropriate organization, thereby *cyclin B1* mRNAs being transported to the animal polar cytoplasm. During this period, translation of *cyclin B1* mRNAs is completely suppressed by the activities of IMP3, CPEB and their co-factors (Fig. 14B and C). In full-grown (stage IV) oocytes, *cyclin B1* mRNAs are stuck in granules that form dense aggregates in the cortical cytoplasm at the animal pole, depending on both Pum1 and the *cis*-acting element in the ORF. This mechanism enables the robust translational repression of *cyclin B1* mRNAs until MIH stimulation. During oocyte maturation after MIH stimulation, *cyclin B1* mRNAs are translationally activated by their dispersion and release from the oocyte cortex due to dissolution of the aggregates (Fig. 14D), which are most likely caused by phosphorylation of Pum1 and CPEB, degradation of CPEB, dissociation of IMP3 and remodeling of cytoskeletons.

References

Abrahamyan LG, Chatel-Chaix L, Ajamian L, Milev MP, Monette A, Clément J-F, Song R, Lehmann M, DesGroseillers L, Laughrea M, Boccaccio G, Mouland AJ. 2010. Novel Staufen1 ribonucleoproteins prevent formation of stress granules but favour encapsidation of HIV-1 genomic RNA. J Cell Sci 123:369–383.

Aguero T, Zhou Y, Kloc M, Chang P, Houliston E, King ML. 2016. Hermes (Rbpms) is a critical component of RNP complexes that sequester germline RNAs during oogenesis. J Dev Biol 4:1699–1712.

Allison R, Czaplinski K, Git A, Adegbenro E, Stennard F, Houliston E, Standart N. 2004. Two distinct Staufen isoforms in Xenopus are vegetally localized during oogenesis. RNA 10:1751–1763.

Arthur PK, Claussen M, Koch S, Tarbashevich K, Jahn O, Pieler T. 2009. Participation of Xenopus Elr-type proteins in vegetal mRNA localization during oogenesis. J Biol Chem 284:19982–19992.

Arumugam K, Wang Y, Hardy LL, MacNicol MC, MacNicol AM. 2010. Enforcing temporal control of maternal mRNA translation during oocyte cell-cycle progression. EMBO J 29:387–397.

Ballantyne S, Daniel DL, Wickens M. 1997. A dependent pathway of cytoplasmic polyadenylation reactions linked to cell cycle control by c-mos and CDK1 activation. Mol Biol Cell 8:1633–1648.

Bally-Cuif L, Schatz WJ, Ho RK. 1998. Characterization of the zebrafish Orb/CPEB-related RNA-binding protein and localization of maternal components in the zebrafish oocyte. Mech Dev 77:31–47.

Barkoff AF, Dickson KS, Gray NK, Wickens M. 2000. Translational control of cyclin B1 mRNA during meiotic maturation: coordinated repression and cytoplasmic polyadenylation. Dev Biol 220:97–109.

Barnard DC, Ryan K, Manley JL, Richter JD. 2004. Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. Cell 119:641–651.

Bateman MJ, Cornell R, D'Alencon C, Sandra A. 2004. Expression of the zebrafish Staufen gene in the embryo and adult. Gene Expr Patterns 5:273–278.

Blackham SL, McGarvey MJ. 2013. A host cell RNA-binding protein, Staufen1, has a role in hepatitis C virus replication before virus assembly. J Gen Virol 94:2429–2436.

Blower MD. 2013. Molecular insights into intracellular RNA localization. Int Rev Cell Mol Biol 302:1–39.

Bondy-Chorney E, Crawford Parks TE, Ravel-Chapuis A, Klinck R, Rocheleau L, Pelchat M, Chabot B, Jasmin BJ, Côté J. 2016. Staufen1 regulates multiple alternative splicing events either positively or negatively in DM1 indicating its role as a disease modifier. PLoS Genet 12:e1005827.

Bontems F, Stein A, Marlow F, Lyautey J, Gupta T, Mullins MC, Dosch R. 2009. Bucky ball organizes germ plasm assembly in zebrafish. Curr Biol 19:414–422.

Boulay K, Ghram M, Viranaicken W, Trépanier V, Mollet S, Fréchina C, DesGroseillers L. 2014. Cell cycle-dependent regulation of the RNA-binding protein Staufen1. Nucleic Acids Res 42:7867–7883.

Braat AK, Zandbergen T, van de Water S, Goos HJ, Zivkovic D. 1999. Characterization of zebrafish primordial germ cells: morphology and early distribution of vasa RNA. Dev Dyn 216:153–167.

Brendza RP, Serbus LR, Duffy JB, Saxton WM. 2000. A function for kinesin I in the posterior transport of oskar mRNA and Staufen protein. Science 289:2120–2122.

Castro A, Peter M, Lorca T, Mandart E. 2001. c-Mos and cyclin B/cdc2 connections during Xenopus oocyte maturation. Biol Cell 93:15–25.

Charlesworth A, Ridge JA, King LA, MacNicol MC, MacNicol AM. 2002. A novel regulatory element determines the timing of Mos mRNA translation during Xenopus oocyte maturation. EMBO J 21:2798–2806.

Charlesworth A, Welk J, Macnicol AM. 2000. The temporal control of Wee1 mRNA translation during Xenopus oocyte maturation is regulated by cytoplasmic polyadenylation elements within the 3'-untranslated region. Dev Biol 227:706–719.

Charlesworth A, Wilczynska A, Thampi P, Cox LL, MacNicol AM. 2006. Musashi regulates the temporal order of mRNA translation during Xenopus oocyte maturation. EMBO J 25:2792–2801.

Charlesworth A, Yamamoto TM, Cook JM, Silva KD, Kotter C V, Carter GS, Holt JW, Lavender HF, MacNicol AM, Ying Wang Y, Wilczynska A. 2012. Xenopus laevis zygote arrest 2 (zar2) encodes a zinc finger RNA-binding protein that binds to the translational control sequence in the maternal Wee1 mRNA and regulates translation. Dev Biol 369:177–190.

Colegrove-Otero LJ, Devaux A, Standart N. 2005. The Xenopus ELAV protein ElrB represses Vg1 mRNA translation during oogenesis. Mol Cell Biol 25:9028–9039.

Cote CA, Gautreau D, Denegre JM, Kress TL, Terry NA, Mowry KL. 1999. A Xenopus protein related to hnRNP I has a role in cytoplasmic RNA localization. Mol Cell 4:431–437.

Czaplinski K, Köcher T, Schelder M, Segref A, Wilm M, Mattaj IW. 2005. Identification of 40LoVe, a Xenopus hnRNP D family protein involved in localizing a TGF-beta-related mRNA during oogenesis. Dev Cell 8:505–515.

Czaplinski K, Mattaj IW. 2006. 40LoVe interacts with Vg1RBP/Vera and hnRNP I in binding the Vg1-localization element. RNA 12:213–222.

de Lucas S, Peredo J, Marión RM, Sánchez C, Ortín J. 2010. Human Staufen1 protein interacts with influenza virus ribonucleoproteins and is required for efficient virus multiplication. J Virol 84:7603–7612.

de Moor CH, Richter JD. 1997. The Mos pathway regulates cytoplasmic polyadenylation in Xenopus oocytes. Mol Cell Biol 17:6419–6426.

de Moor CH, Richter JD. 1999. Cytoplasmic polyadenylation elements mediate masking and unmasking of cyclin B1 mRNA. EMBO J 18:2294–2303.

Deshler JO, Highett MI, Abramson T, Schnapp BJ. 1998. A highly conserved RNA-binding protein for cytoplasmic mRNA localization in vertebrates. Curr Biol 8:489–496.

Deshler JO, Highett MI, Schnapp BJ. 1997. Localization of Xenopus Vg1 mRNA by Vera protein and the endoplasmic reticulum. Science 276:1128–1131.

Dosch R, Wagner DS, Mintzer KA, Runke G, Wiemelt AP, Mullins MC. 2004. Maternal control of vertebrate development before the midblastula transition: mutants from the zebrafish I. Dev Cell 6:771–780.

Dugré-Brisson S, Elvira G, Boulay K, Chatel-Chaix L, Mouland AJ, DesGroseillers L. 2005. Interaction of Staufen1 with the 5’ end of mRNA facilitates translation of these RNAs. Nucleic Acids Res 33:4797–4812.

Dumont JN. 1972. Oogenesis in Xenopus laevis (Daudin). I. Stages of oocyte development in laboratory maintained animals. J Morphol 136:153–179.

Elisha Z, Havin L, Ringel I, Yisraeli JK. 1995. Vg1 RNA binding protein mediates the association of Vg1 RNA with microtubules in Xenopus oocytes. EMBO J 14:5109–5114.

Ephrussi A, Lehmann R. 1992. Induction of germ cell formation by oskar. Nature 358:387–392.

Ferrandon D, Elphick L, Nüsslein-Volhard C, St Johnston D. 1994. Staufen protein associates with the 3’UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell 79:1221–1232.

Ford LP, Watson J, Keene JD, Wilusz J. 1999. ELAV proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system. Genes Dev 13:188–201.

Gautrey H, McConnell J, Lako M, Hall J, Hesketh J. 2008. Staufen1 is expressed in preimplantation mouse embryos and is required for embryonic stem cell differentiation. Biochim Biophys Acta 1783:1935–1942.

Git A, Allison R, Perdiguero E, Nebreda AR, Houliston E, Standart N. 2009. Vg1RBP phosphorylation by Erk2 MAP kinase correlates with the cortical release of Vg1 mRNA during meiotic maturation of Xenopus oocytes. RNA 15:1121–1133.

Git A, Standart N. 2002. The KH domains of Xenopus Vg1RBP mediate RNA binding and self-association. RNA 8:1319–1333.

Haccard O, Jessus C. 2006. Oocyte maturation, Mos and cyclins--a matter of synthesis: two functionally redundant ways to induce meiotic maturation. Cell Cycle 5:1152–1159.

Heim AE, Hartung O, Rothhämel S, Ferreira E, Jenny A, Marlow FL. 2014. Oocyte polarity requires a Bucky ball-dependent feedback amplification loop. Development 141:842–854.

Hirai T, Yamashita M, Yoshikuni M, Tokumoto T, Kajiura H, Sakai N, Nagahama Y. 1992. Isolation and characterization of goldfish cdk2, a cognate variant of the cell cycle regulator cdc2. Dev Biol 152:113–120.

Houston DW. 2013. Regulation of cell polarity and RNA localization in vertebrate oocytes. Int Rev Cell Mol Biol 306:127–185.

Howley C, Ho RK. 2000. mRNA localization patterns in zebrafish oocytes. Mech Dev 92:305–309.

Ihara J, Yoshida N, Tanaka T, Mita K, Yamashita M. 1998. Either cyclin B1 or B2 is necessary and sufficient for inducing germinal vesicle breakdown during frog (Rana japonica) oocyte maturation. Mol Reprod Dev 50:499–509.

Katsu Y, Yamashita M, Kajiura H, Nagahama Y. 1993. Behavior of the components of maturation-promoting factor, cdc2 kinase and cyclin B, during oocyte maturation of goldfish. Dev Biol 160:99–107.

Kiebler MA, Hemraj I, Verkade P, Köhrmann M, Fortes P, Marión RM, Ortín J, Dotti CG. 1999. The mammalian staufen protein localizes to the somatodendritic domain of cultured hippocampal neurons: implications for its involvement in mRNA transport. J Neurosci 19:288–297.

Kim JH, Richter JD. 2006. Opposing polymerase-deadenylase activities regulate cytoplasmic polyadenylation. Mol Cell 24:173–183.

King ML, Messitt TJ, Mowry KL. 2005. Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. Biol Cell 97:19–33.

Kloc M, Etkin LD. 1995. Two distinct pathways for the localization of RNAs at the vegetal cortex in Xenopus oocytes. Development 121:287–297.

Kloc M, Etkin LD. 2005. RNA localization mechanisms in oocytes. J Cell Sci 118:269–282.

Köhrmann M, Luo M, Kaether C, DesGroseillers L, Dotti CG, Kiebler MA. 1999. Microtubule-dependent recruitment of Staufen-green fluorescent protein into large RNA-containing granules and subsequent dendritic transport in living hippocampal neurons. Mol Biol Cell 10:2945–2953.

Kolev NG, Huber PW. 2003. VgRBP71 stimulates cleavage at a polyadenylation signal in Vg1 mRNA, resulting in the removal of a cis-acting element that represses translation. Mol Cell 11:745–755.

Kondo T, Kotani T, Yamashita M. 2001. Dispersion of cyclin B mRNA aggregation is coupled with translational activation of the mRNA during zebrafish oocyte maturation. Dev Biol 229:421–431.

Kondo T, Yanagawa T, Yoshida N, Yamashita M. 1997. Introduction of cyclin B induces activation of the maturation-promoting factor and breakdown of germinal vesicle in

growing zebrafish oocytes unresponsive to the maturation-inducing hormone. Dev Biol 190:142–152.

Kosaka K, Kawakami K, Sakamoto H, Inoue K. 2007. Spatiotemporal localization of germ plasm RNAs during zebrafish oogenesis. Mech Dev 124:279–289.

Kotani T, Yasuda K, Ota R, Yamashita M. 2013. Cyclin B1 mRNA translation is temporally controlled through formation and disassembly of RNA granules. J Cell Biol 202:1041–1055.

Kress TL, Yoon YJ, Mowry KL. 2004. Nuclear RNP complex assembly initiates cytoplasmic RNA localization. J Cell Biol 165:203–211.

Kroll TT, Swenson LB, Hartland EI, Snedden DD, Goodson H V, Huber PW. 2009. Interactions of 40LoVe within the ribonucleoprotein complex that forms on the localization element of Xenopus Vg1 mRNA. Mech Dev 126:523–538.

Kroll TT, Zhao W-M, Jiang C, Huber PW. 2002. A homolog of FBP2/KSRP binds to localized mRNAs in Xenopus oocytes. Development 129:5609–5619.

Ku M, Melton DA. 1993. Xwnt-11: a maternally expressed Xenopus wnt gene. Development 119:1161–1173.

Kumano G. 2012. Polarizing animal cells via mRNA localization in oogenesis and early development. Dev Growth Differ 54:1–18.

Kwon S, Abramson T, Munro TP, John CM, Köhrmann M, Schnapp BJ. 2002. UUCAC- and vera-dependent localization of VegT RNA in Xenopus oocytes. Curr Biol 12:558–564.

Lederer M, Bley N, Schleifer C, Hüttelmaier S. 2014. The role of the oncofetal IGF2 mRNA-binding protein 3 (IGF2BP3) in cancer. Semin Cancer Biol 29:3–12.

Liao B, Hu Y, Herrick DJ, Brewer G. 2005. The RNA-binding protein IMP-3 is a translational activator of insulin-like growth factor II leader-3 mRNA during proliferation of human K562 leukemia cells. J Biol Chem 280:18517–18524.

Lin C-L, Evans V, Shen S, Xing Y, Richter JD. 2010. The nuclear experience of CPEB: implications for RNA processing and translational control. RNA 16:338–348.

MacNicol MC, MacNicol AM. 2010. Developmental timing of mRNA translation-integration of distinct regulatory elements. Mol Reprod Dev 77:662–669.

Marlow FL, Mullins MC. 2008. Bucky ball functions in Balbiani body assembly and animal-vegetal polarity in the oocyte and follicle cell layer in zebrafish. Dev Biol 321:40–50.

Martin KC, Ephrussi A. 2009. mRNA localization: gene expression in the spatial dimension. Cell 136:719–730.

Mendez R, Richter JD. 2001. Translational control by CPEB: a means to the end. Nat Rev Mol Cell Biol 2:521–529.

Messitt TJ, Gagnon JA, Kreiling JA, Pratt CA, Yoon YJ, Mowry KL. 2008. Multiple kinesin motors coordinate cytoplasmic RNA transport on a subpopulation of microtubules in Xenopus oocytes. Dev Cell 15:426–436.

Mimori-Kiyosue Y, Shiina N, Tsukita S. 2000. The dynamic behavior of the APC-binding protein EB1 on the distal ends of microtubules. Curr Biol 10:865–868.

Mowry KL, Cote CA. 1999. RNA sorting in Xenopus oocytes and embryos. FASEB J 13:435–445.

Mowry KL, Melton DA. 1992. Vegetal messenger RNA localization directed by a 340-nt RNA sequence element in Xenopus oocytes. Science 255:991–994.

Murakami MS, Vande Woude GF. 1998. Analysis of the early embryonic cell cycles of Xenopus; regulation of cell cycle length by Xe-wee1 and Mos. Development 125:237–248.

Nagahama Y, Yamashita M. 2008. Regulation of oocyte maturation in fish. Dev Growth Differ 50:S195-219.

Nair S, Lindeman RE, Pelegri F. 2013. In vitro oocyte culture-based manipulation of zebrafish maternal genes. Dev Dyn 242:44–52.

Nakahata S, Katsu Y, Mita K, Inoue K, Nagahama Y, Yamashita M. 2001. Biochemical identification of Xenopus Pumilio as a sequence-specific cyclin B1 mRNA-binding protein that physically interacts with a Nanos homolog, Xcat-2, and a cytoplasmic polyadenylation element-binding protein. J Biol Chem 276:20945–20953.

Nakahata S, Kotani T, Mita K, Kawasaki T, Katsu Y, Nagahama Y, Yamashita M. 2003. Involvement of Xenopus Pumilio in the translational regulation that is specific to cyclin B1 mRNA during oocyte maturation. Mech Dev 120:865–880.

Nakajo N, Yoshitome S, Iwashita J, Iida M, Uto K, Ueno S, Okamoto K, Sagata N. 2000. Absence of Wee1 ensures the meiotic cell cycle in Xenopus oocytes. Genes Dev 14:328–338.

Nebreda AR, Ferby I. 2000. Regulation of the meiotic cell cycle in oocytes. Curr Opin Cell Biol 12:666–675.

Nielsen J, Christiansen J, Lykke-Andersen J, Johnsen AH, Wewer UM, Nielsen FC. 1999. A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. Mol Cell Biol 19:1262–1270.

Nukada Y, Horie M, Fukui A, Kotani T, Yamashita M. 2015. Real-time imaging of actin filaments in the zebrafish oocyte and embryo. Cytoskeleton 72:491–501.

Ota R, Kotani T, Yamashita M. 2011a. Biochemical characterization of Pumilio1 and Pumilio2 in Xenopus oocytes. J Biol Chem 286:2853–2863.

Ota R, Kotani T, Yamashita M. 2011b. Possible involvement of Nemo-like kinase 1 in Xenopus oocyte maturation as a kinase responsible for Pumilio1, Pumilio2, and CPEB phosphorylation. Biochemistry 50:5648–5659.

Ota R, Suwa K, Kotani T, Mita K, Yamashita M. 2008. Possible involvement of phosphatidylinositol 3-kinase, but not protein kinase B or glycogen synthase kinase 3beta, in progesterone-induced oocyte maturation in the Japanese brown frog, Rana japonica. Zoolog Sci 25:773–781.

Otero LJ, Devaux A, Standart N. 2001. A 250-nucleotide UA-rich element in the 3' untranslated region of Xenopus laevis Vg1 mRNA represses translation both in vivo and in vitro. RNA 7:1753–1767.

Park E, Maquat LE. 2013. Staufen-mediated mRNA decay. Wiley Interdiscip Rev RNA 4:423–435.

Peng SS, Chen CY, Xu N, Shyu AB. 1998. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J 17:3461–3470.

Piqué M, López JM, Foissac S, Guigó R, Méndez R. 2008. A combinatorial code for CPE-mediated translational control. Cell 132:434–448.

Radford HE, Meijer HA, de Moor CH. 2008. Translational control by cytoplasmic polyadenylation in Xenopus oocytes. Biochim Biophys Acta 1779:217–229.

Ramasamy S, Wang H, Quach HNB, Sampath K. 2006. Zebrafish Staufen1 and Staufen2 are required for the survival and migration of primordial germ cells. Dev Biol 292:393–406.

Ravel-Chapuis A, Bélanger G, Yadava RS, Mahadevan MS, DesGroseillers L, Côté J, Jasmin BJ. 2012. The RNA-binding protein Staufen1 is increased in DM1 skeletal muscle and promotes alternative pre-mRNA splicing. J Cell Biol 196:699–712.

Richter JD. 2007. CPEB: a life in translation. Trends Biochem Sci 32:279–285.

Schulte-Merker S, Ho RK, Herrmann BG, Nüsslein-Volhard C. 1992. The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development 116:1021–1032.

Schwartz SP, Aisenthal L, Elisha Z, Oberman F, Yisraeli JK. 1992. A 69-kDa RNA-binding protein from Xenopus oocytes recognizes a common motif in two vegetally localized maternal mRNAs. Proc Natl Acad Sci U S A 89:11895–11899.

Seki S, Kouya T, Tsuchiya R, Valdez DM, Jin B, Hara T, Saida N, Kasai M, Edashige K. 2008. Development of a reliable in vitro maturation system for zebrafish oocytes. Reproduction 135:285–292.

Selman K, Wallace RA, Sarka A, Qi X. 1993. Stages of oocyte development in the zebrafish, Brachydanio rerio. J Morphol 218:203–224.

Sheets MD, Fox CA, Hunt T, Vande Woude G, Wickens M. 1994. The 3'-untranslated regions of c-mos and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. Genes Dev 8:926–938.

St Johnston D, Beuchle D, Nüsslein-Volhard C. 1991. Staufen, a gene required to localize maternal RNAs in the Drosophila egg. Cell 66:51–63.

St Johnston D, Driever W, Berleth T, Richstein S, Nüsslein-Volhard C. 1989. Multiple steps in the localization of bicoid RNA to the anterior pole of the Drosophila oocyte. Development 107 Suppl:13–19.

Suzuki H, Maegawa S, Nishibu T, Sugiyama T, Yasuda K, Inoue K. 2000. Vegetal localization of the maternal mRNA encoding an EDEN-BP/Bruno-like protein in zebrafish. Mech Dev 93:205–209.

Suzuki H, Tsukahara T, Inoue K. 2009. Localization of c-mos mRNA around the animal pole in the zebrafish oocyte with Zor-1/Zorba. Biosci Trends 3:96–104.

Tanaka T, Yamashita M. 1995. Pre-MPF is absent in immature oocytes of fishes and amphibians except Xenopus. Dev Growth Differ 37:387–393.

Tao Q, Yokota C, Puck H, Kofron M, Birsoy B, Yan D, Asashima M, Wylie CC, Lin X, Heasman J. 2005. Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. Cell 120:857–871.

Thomas MG, Martinez Tosar LJ, Desbats MA, Leishman CC, Boccaccio GL. 2009. Mammalian Staufen 1 is recruited to stress granules and impairs their assembly. J Cell Sci 122:563–573.

Vikesaa J, Hansen TVO, Jønson L, Borup R, Wewer UM, Christiansen J, Nielsen FC. 2006. RNA-binding IMPs promote cell adhesion and invadopodia formation. EMBO J 25:1456–1468.

Wang W, Caldwell MC, Lin S, Furneaux H, Gorospe M. 2000. HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. EMBO J 19:2340–2350.

Wang YY, Charlesworth A, Byrd SM, Gregerson R, MacNicol MC, MacNicol AM. 2008. A novel mRNA 3’ untranslated region translational control sequence regulates Xenopus Wee1 mRNA translation. Dev Biol 317:454–466.

Yamamoto TM, Cook JM, Kotter C V, Khat T, Silva KD, Ferreyros M, Holt JW, Knight JD, Charlesworth A. 2013. Zar1 represses translation in Xenopus oocytes and binds to the TCS in maternal mRNAs with different characteristics than Zar2. Biochim Biophys Acta 1829:1034–1046.

Yamashita M. 1998. Molecular mechanisms of meiotic maturation and arrest in fish and amphibian oocytes. Semin Cell Dev Biol 9:569–579.

Yamashita M. 2000. Toward modeling of a general mechanism of MPF formation during oocyte maturation in vertebrates. Zoolog Sci 17:841–851.

Yamashita M, Yoshikuni M, Hirai T, Fukada S, Nagahama Y. 1991. A monoclonal antibody against the PSTAIR sequence of p34cdc2, catalytic subunit of maturation ‐promoting factor and key regulator of the cell cycle. Dev Growth Differ 33:617–624.

Yaniv K, Yisraeli JK. 2002. The involvement of a conserved family of RNA binding proteins in embryonic development and carcinogenesis. Gene 287:49–54.

Yasuda K, Kotani T, Ota R, Yamashita M. 2010. Transgenic zebrafish reveals novel mechanisms of translational control of cyclin B1 mRNA in oocytes. Dev Biol 348:76–86.

Yasuda K, Kotani T, Yamashita M. 2013. A cis-acting element in the coding region of cyclin B1 mRNA couples subcellular localization to translational timing. Dev Biol 382:517–529.

Yisraeli JK. 2005. VICKZ proteins: a multi-talented family of regulatory RNA-binding proteins. Biol Cell 97:87–96.

Yisraeli JK, Sokol S, Melton DA. 1990. A two-step model for the localization of maternal mRNA in Xenopus oocytes: involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA. Development 108:289–298.

Yoon YJ, Mowry KL. 2004. Xenopus Staufen is a component of a ribonucleoprotein complex containing Vg1 RNA and kinesin. Development 131:3035–3045.

Zhang Y, Sheets MD. 2009. Analyses of zebrafish and Xenopus oocyte maturation reveal conserved and diverged features of translational regulation of maternal cyclin B1 mRNA. BMC Dev Biol 9:7.

Zhao WM, Jiang C, Kroll TT, Huber PW. 2001. A proline-rich protein binds to the localization element of Xenopus Vg1 mRNA and to ligands involved in actin polymerization. EMBO J 20:2315–2325.

Zhou Y, King ML. 1996. Localization of Xcat-2 RNA, a putative germ plasm component, to the mitochondrial cloud in Xenopus stage I oocytes. Development 122:2947–2953.

Table 1. Primer sequences used in Chapter I

Purpose	Name/ Target		Sequence (5'-3')
Construction	IMP3_ORF_F		atcgatATGAATAAGCTGTACATCGGGAA
	IMP3_ORF_R		ctcgagTTTCCTCCTGGCGACTG
	IMP3_ex_F		caccCTACGCTTGCTGGTACCG
	IMP3_ex_R		TTCCGACTCCATCTGCG
	GFCPEB_ORF_F		GAagatctATGGCGTTTTCTCTGAGC
	cyclin B1	Forward	TTGGGGTTATGCTG
		Reverse	AAAACTTTAAAAAGTTTATTTGAA
	β-globin	Forward	AGTCTCATCGCCAATGAACG
		Reverse	GCTTTTAACATTATTTTATTGAT
RT-PCR	*cyclin B1*	Forward	GAGGGCCTTTCTAAGCATCTGGCTGTG
		Reverse	TTATTTGAATTCAAATGTACAAACTTGC
	mos	Forward	TATAACCTGCGCCCTTTGACCAGC
		Reverse	ACATTTTTGCATAAAAAATTTAGCTTCAC
	wee1	Forward	TTTTATCCATCCAAGCAAGCGAGC
		Reverse	TTTACAAACAAAGAGTTAACAAGACC
	β-actin	Forward	GGTAGTTGTCTAACAGGGGAGAGC
		Reverse	GTTGACTTGTCAGTGTACAGAGA

Additional sequences used for cloning are indicated by lowercase letters.

Table 2. Primer sequences used in Chapter II

Purpose	Name/ Target		Sequence (5'-3')
Construction	Stau1_exF		caccAACCCCATCAGCAGACTGG
	Stau1_exR		GGCTGTCCCATCCAGAGTC
	Stau1_IF_F		ccatcgattcgaattTGGCAAACCCTAAAGAGAAGACC
	Stau1_IF_R		gagaggccttgaattCTAGGCTGTCCCATCCAGAGTC
	xVg1VLE_IF_F		aagcagatctcatagACTTTATTTCTACACTGTTATGTC
	xVg1VLE_IF_R		tatcttcgcagatctGCTCAAGTCATATGGACTATTATAT
	cyclinB1_ORF_R		CTATGAGATCTGCTTAGCCAG
	pT2KXIGΔin_BglII_F		AGATCTGCGAAGATACGG
	EB1_exF		caccATGGCTGTGAACGTATATTC
	EB1_exR		GAACTCCTCCTGGTCC
RT-PCR	*cyclin B1*	Forward	GACAGGCTTTGAAGAAGAAGGAGG
		Reverse	GGAAGGCTCAGACACAACCTTAA
	zorba	Forward	GGCATCGACACAGACAAGC
		Reverse	CCAGGTTCACGGCTGCATG
	mos	Forward	CGTAATGGAGTTCGCAGGCAATA
		Reverse	TCTGACAACAAGACATTGGCTGG
	vg1	Forward	TACGAGGAAACATCGTGAGG
		Reverse	CGTTACTCCCTTCAGTGTGG
	dazl	Forward	CCAGAAGCATCGTCAGGG
		Reverse	GTCTGGATATCAACATCCTCACTG
	brul	Forward	GACTCGGAGATGCTGCAGATG
		Reverse	GATCTGAAAGCCGTTCATGG
	vasa	Forward	GACATGCCCAGCAGCATCG
		Reverse	GGAACTACTTGTTGGGCCCC
	buc	Forward	GAGGAGTATGGTGCTCTGGC
		Reverse	GAGTTCTCCCTTGATCCTGAG
	actin	Forward	AAATCGCTGCCCTGGTCGTT
		Reverse	CTGTCCCATGCCAACCATCA
	gfp	Forward	CGACCACTACCAGCAGAACA
		Reverse	ACGAACTCCAGCAGGACCAT

Additional sequences used for cloning are indicated by lowercase letters.

Fig. 1

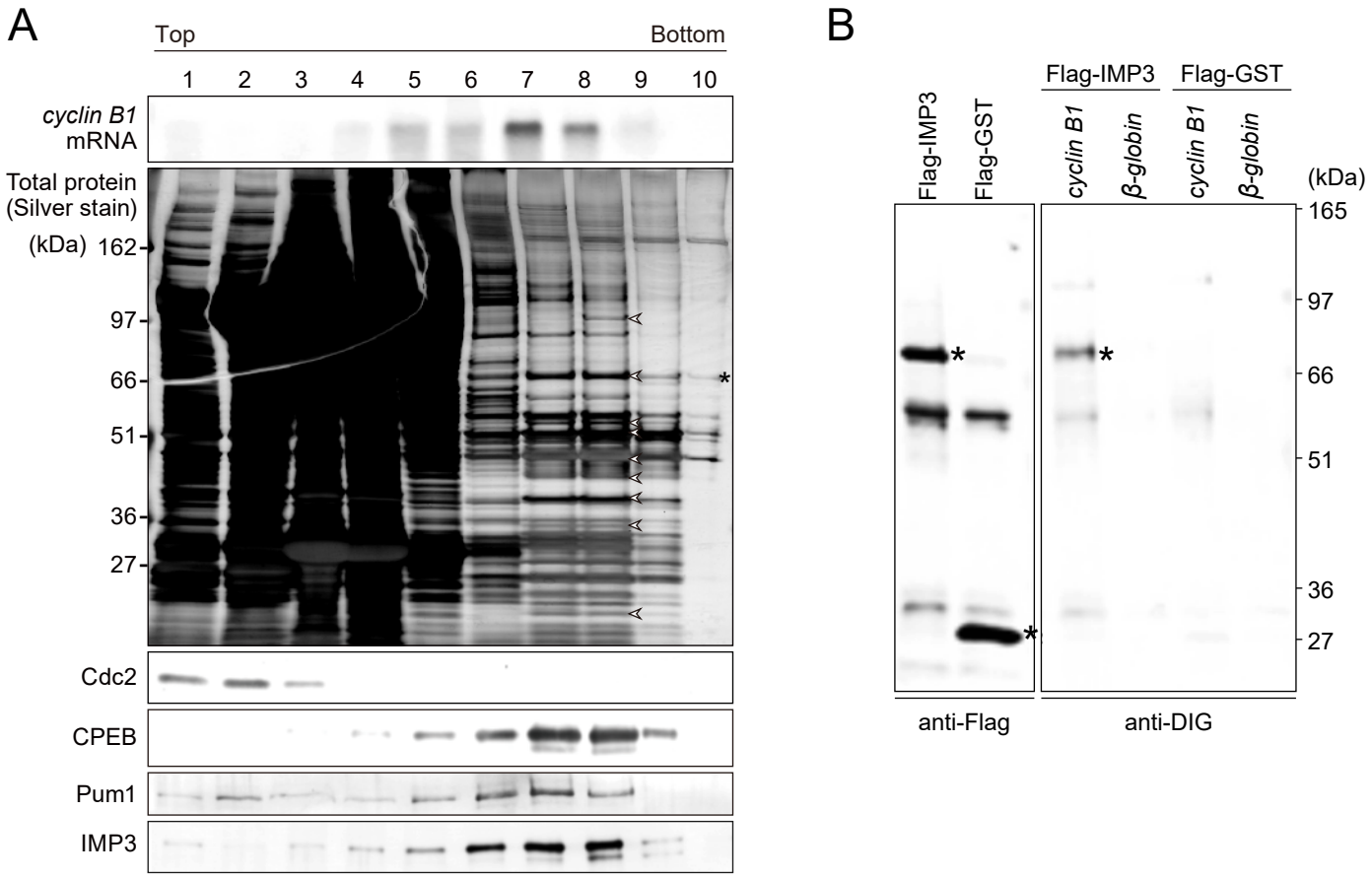

Fig. 1. Identification of IMP3 as a *cyclin B1* mRNA-binding protein.

(A) Fractionation by sucrose density gradient ultracentrifugation. The distribution of *cyclin B1* mRNA was analyzed by northern blotting, and the distribution of total protein was analyzed by SDS-PAGE followed by silver staining. Arrowheads on the silver-stained gel indicate the bands analyzed by mass spectrometry, and an asterisk indicates the band of IMP3. Cdc2, CPEB, Pum1 and IMP3 in each fraction were analyzed by immunoblotting.

(B) UV cross-linking assay. Flag-tagged IMP3 and GST are shown with anti-Flag antibody. Flag-tagged proteins were incubated with DIG-labeled *cyclin B1* or *β-globin* probes, and the bound probes were detected with anti-DIG antibody. Asterisks indicate specific signals.

Fig. 2

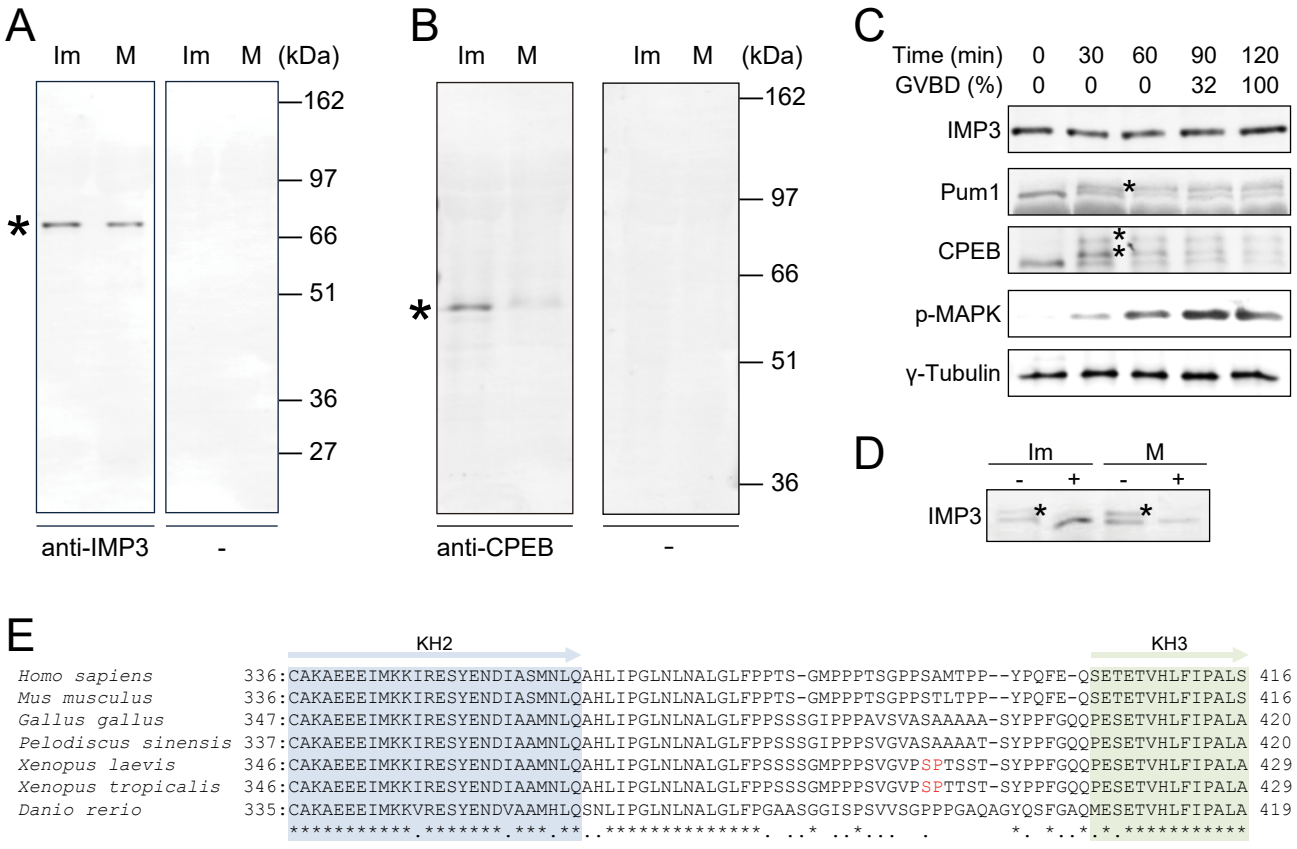

Fig. 2. Characterization of anti-IMP3 and anti-CPEB antibodies, and behaviors of key proteins during oocyte maturation

(A) Immunoblotting of immature (Im) and mature (M) oocyte extracts with anti-IMP3 antibody or without a primary antibody (-). Asterisk indicates the anti-IMP3-positive 69-kDa protein.

(B) Experiments similar to those in (A) using anti-CPEB antibody. Asterisk indicates the anti-CPEB-positive 60-kDa protein.

(C) MIH-treated oocytes were analyzed with anti-IMP3, anti-Pum1, anti-CPEB, anti-phospho-MAPK or anti-γ-tubulin antibodies. Asterisks indicate phosphorylated Pum1 and CPEB.

(D) Immature (Im) and mature (M) oocyte extracts were immunoprecipitated with anti-IMP3 antibody. The resulting precipitates were incubated with (+) or without (-) λ-phosphatase and analyzed by Phos-tag SDS-PAGE followed by immunoblotting with anti-IMP3 antibody. Asterisks indicate phosphorylated IMP3.

(E) Alignment of a sequence around S402 and P403 in the frog *Xenopus laevis* IMP3 (GenBank accession: NP_001081752) with the corresponding sequences in the frog *Xenopus 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.

Fig. 3

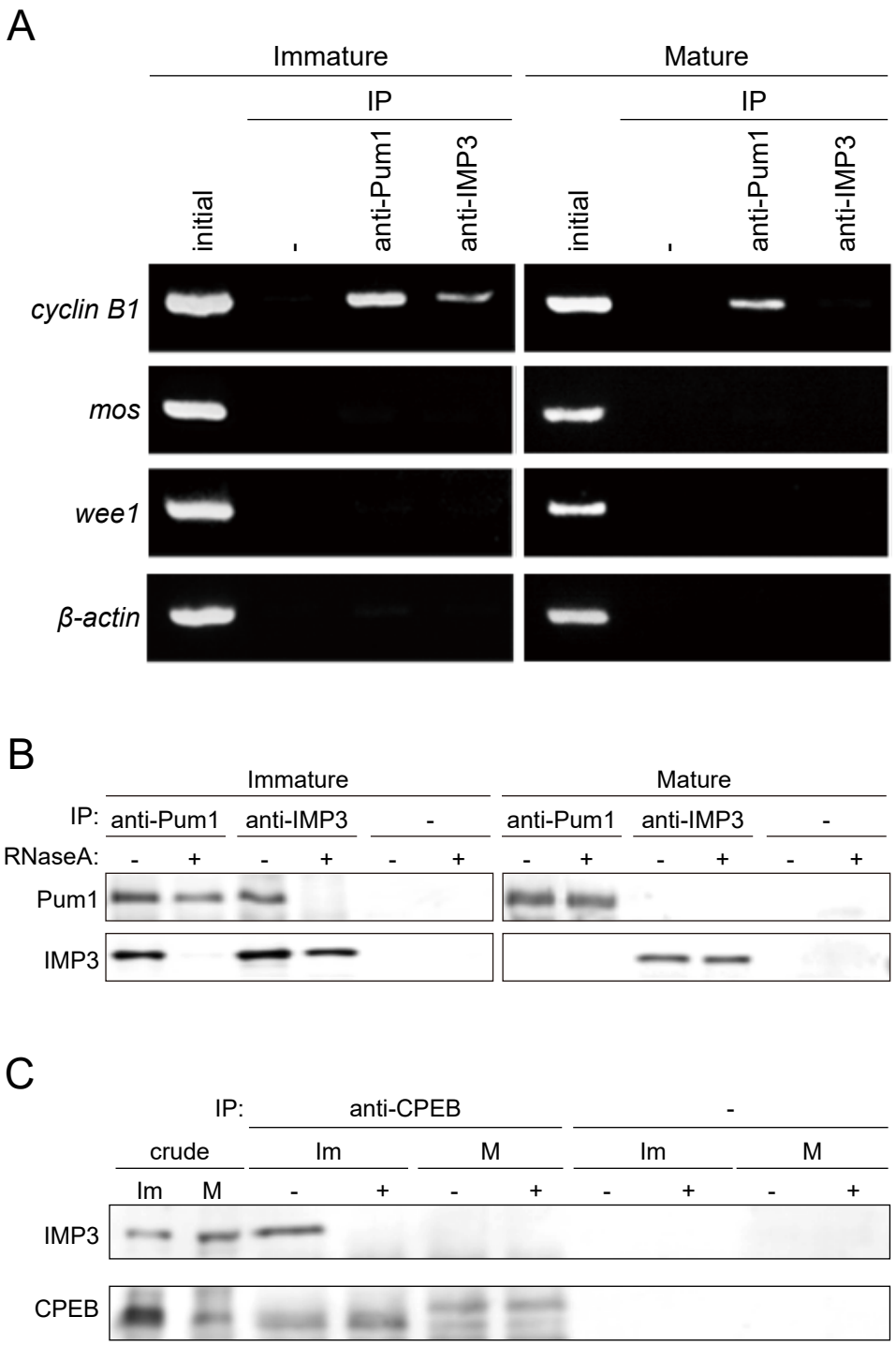

Fig. 3. Interaction of IMP3 with *cyclin B1* mRNA, Pumilio1 and CPEB.

(A) Immature and mature oocyte extracts were subjected to immunoprecipitation (IP) with anti-Pum1, anti-IMP3 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-Pum1, anti-IMP3 or without an antibody (-). 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. 4

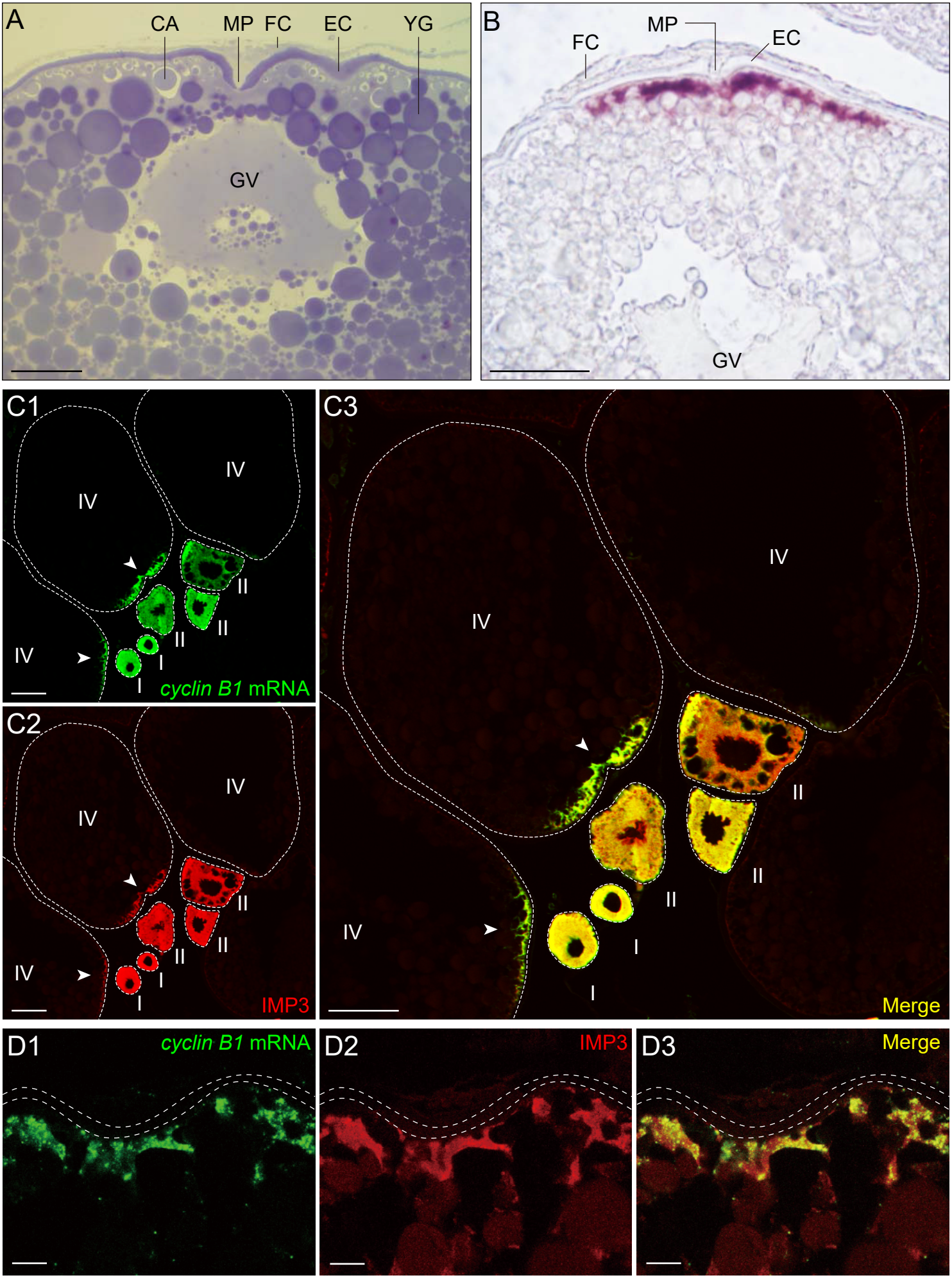
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Fig. 4. Subcellular localization of IMP3 and *cyclin B1* mRNA in immature oocytes.

(A) Morphology of the animal polar region of immature zebrafish oocytes. An Epon thick section stained with toluidine blue.

(B) *In situ* hybridization of *cyclin B1* mRNA. Purple signals show *cyclin B1* mRNAs localized to the animal polar cytoplasm.

(C) *In situ* hybridization of *cyclin B1* mRNA (C1: green), immunostaining of IMP3 (C2: red), and merged (C3) images are shown. The egg chorion is outlined by broken lines, and the stages of oocytes are indicated by Roman numerals. *cyclin B1* mRNAs localized to the animal polar cytoplasm are indicated by arrowheads.

(D) Higher magnification of the animal polar region of stage IV oocyte. *In situ* hybridization of *cyclin B1* mRNA (D1: green), immunostaining of IMP3 (D2: red), and merged (D3) images are shown.

CA, cortical alveolus; EC, egg chorion; FC, follicle cell; GV, germinal vesicle; MP, micropyle; YG, yolk granule. Scale bars: (A and B) 50 μm, (C) 100 μm, (D) 10 μm.

Fig. 5

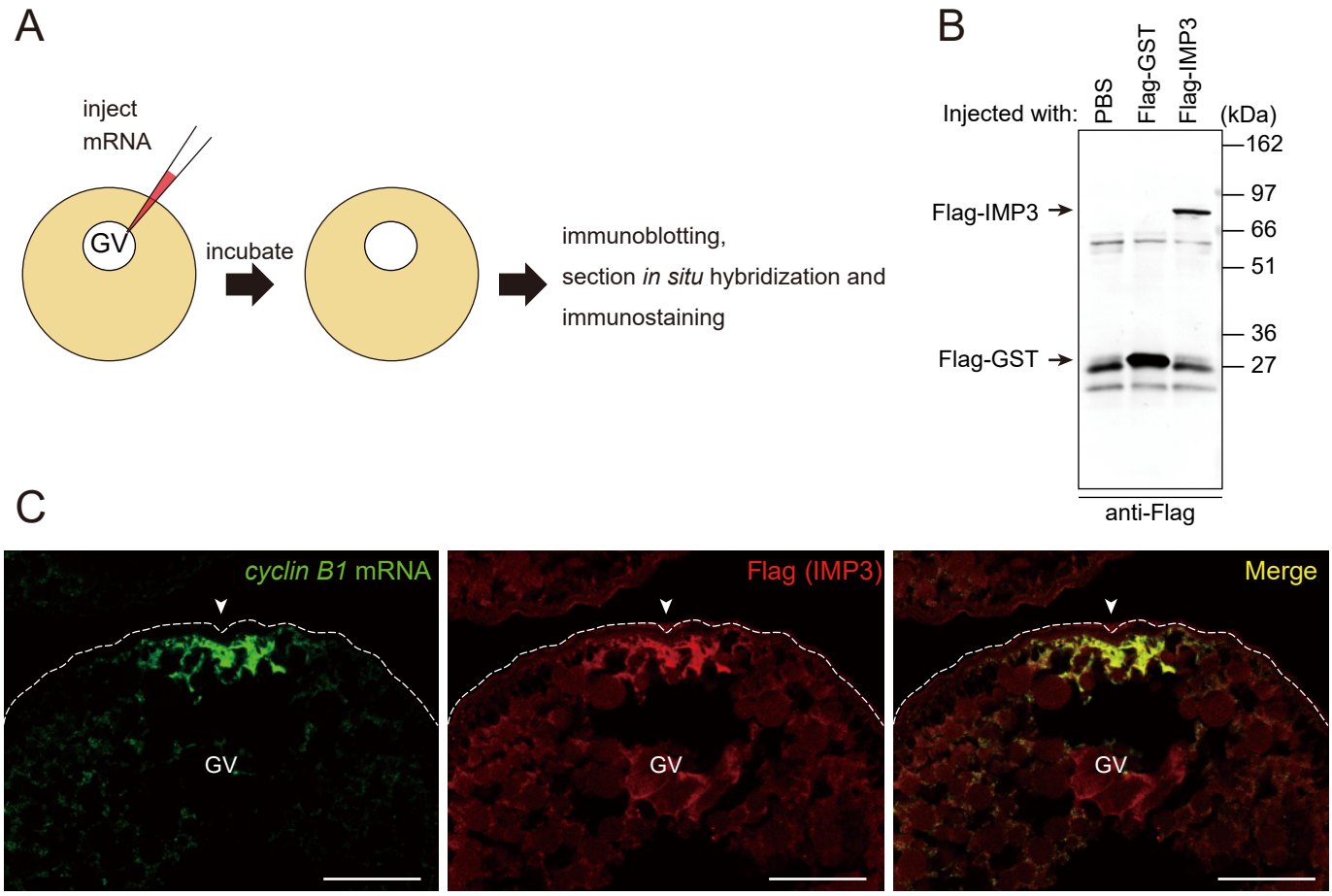

Fig. 5. Overexpression of Flag-IMP3 in full-grown oocytes.

(A) Schematic representation of the experimental procedure. PBS or mRNAs encoding *Flag-GST* or *Flag-IMP3* were injected into the nucleus and incubated for 2 hr to allow protein expression. The oocytes were then prepared for immunoblotting (B), and fixed for section *in situ* hybridization and immunostaining (C).

(B) Oocytes injected with PBS or mRNAs encoding *Flag-GST* or *Flag-IMP3* were examined by immunoblotting with anti-Flag antibody to detect the protein expression from each mRNA. Arrows indicate specific signals.

(C) Subcellular localization of *cyclin B1* mRNA and Flag-IMP3 in full-grown oocytes injected with *Flag-IMP3* mRNA. *In situ* hybridization of *cyclin B1* mRNA (green), immunostaining of Flag (red) and merged images are shown. GV, germinal vesicle; Arrowheads, animal pole; Scale bars, 100 μm.

Fig. 6

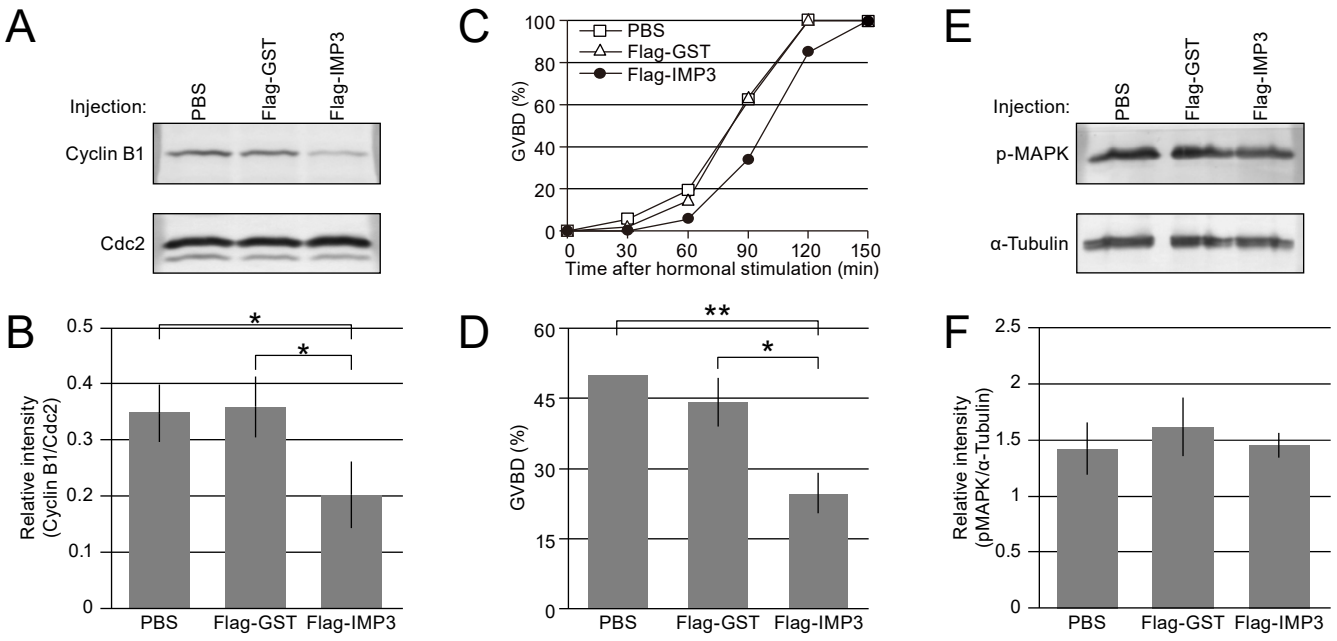

Fig. 6. Effects of overexpression of Flag-IMP3 on translation of *cyclin B1* mRNA and oocyte maturation.

(A) Immunoblotting of Suc1-precipitates from injected oocytes with anti-Cyclin B1 or anti-Cdc2. The upper bands of Cdc2 are inactive form, whereas the lower bands are active form.

(B) Relative amounts of Cyclin B1 proteins compaired to inactive Cdc2 proteins in (A). Values are means ± S.D. for three independent experiments. Statistical analysis was performed by two-tailed unpaired Student's *t*-test (**P* < 0.05).

(C) Time course of GVBD in oocytes injected with PBS or mRNAs encoding *Flag-GST* or *Flag-IMP3.* Representative results are shown.

(D) Percentage of GVBD in the *Flag-GST* or *Flag-IMP3* mRNA-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 two-tailed unpaired Student's *t*-test (**P* < 0.05, ***P* < 0.005).

(E) Immunoblotting of crude extracts from injected oocytes with anti-phospho-MAPK or anti-α-Tubulin.

(F) Relative amounts of phospho-MAPK proteins compaired to α-Tubulin proteins in (C). Values are means ± S.D. for three independent experiments.

Fig. 7

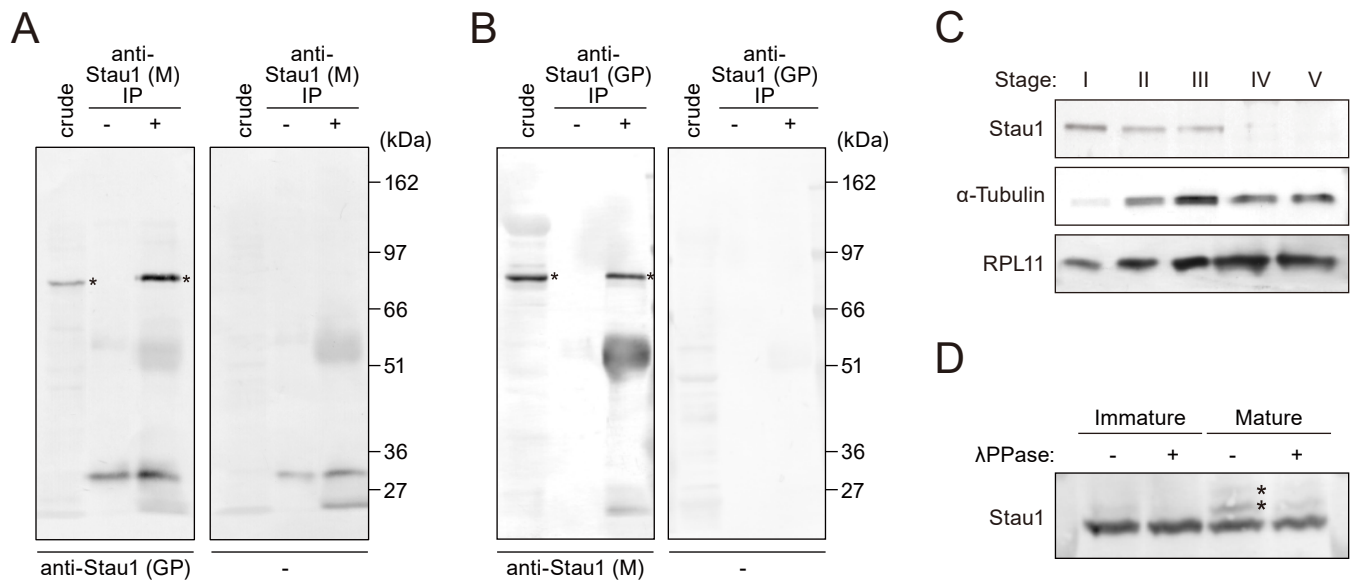

Fig. 7. Staufen1 protein (Stau1) during oocyte growth and maturation in zebrafish.

(A) Crude extracts (10 μg, crude) and immunoprecipitates (IP) with (+) or without (-) anti-Stau1 (M) antibody from stages I and II oocytes were immunoblotted with anti-Stau1 (GP) antibody or without the first antibody. Asterisks indicate Stau1.

(B) Experiments similar to those in (A) using anti-Stau1 (GP) for immunoprecipitation and anti-Stau1 (M) for immunoblotting.

(C) Oocyte extracts equivalent to a single oocyte of stages I to V were analyzed by immunoblotting with anti-Stau1 (GP), anti-α-Tubulin or anti-RPL11 antibodies.

(D) Anti-Stau1 (GP) immunoprecipitates from immature (stage IV) and mature (stage V) oocyte extracts were treated with (+) or without (-) λ-phosphatase and analyzed by Phos-tag SDS–PAGE followed by anti-Stau1 (M) immunoblotting. Asterisks indicate phosphorylated Stau1.

Fig. 8

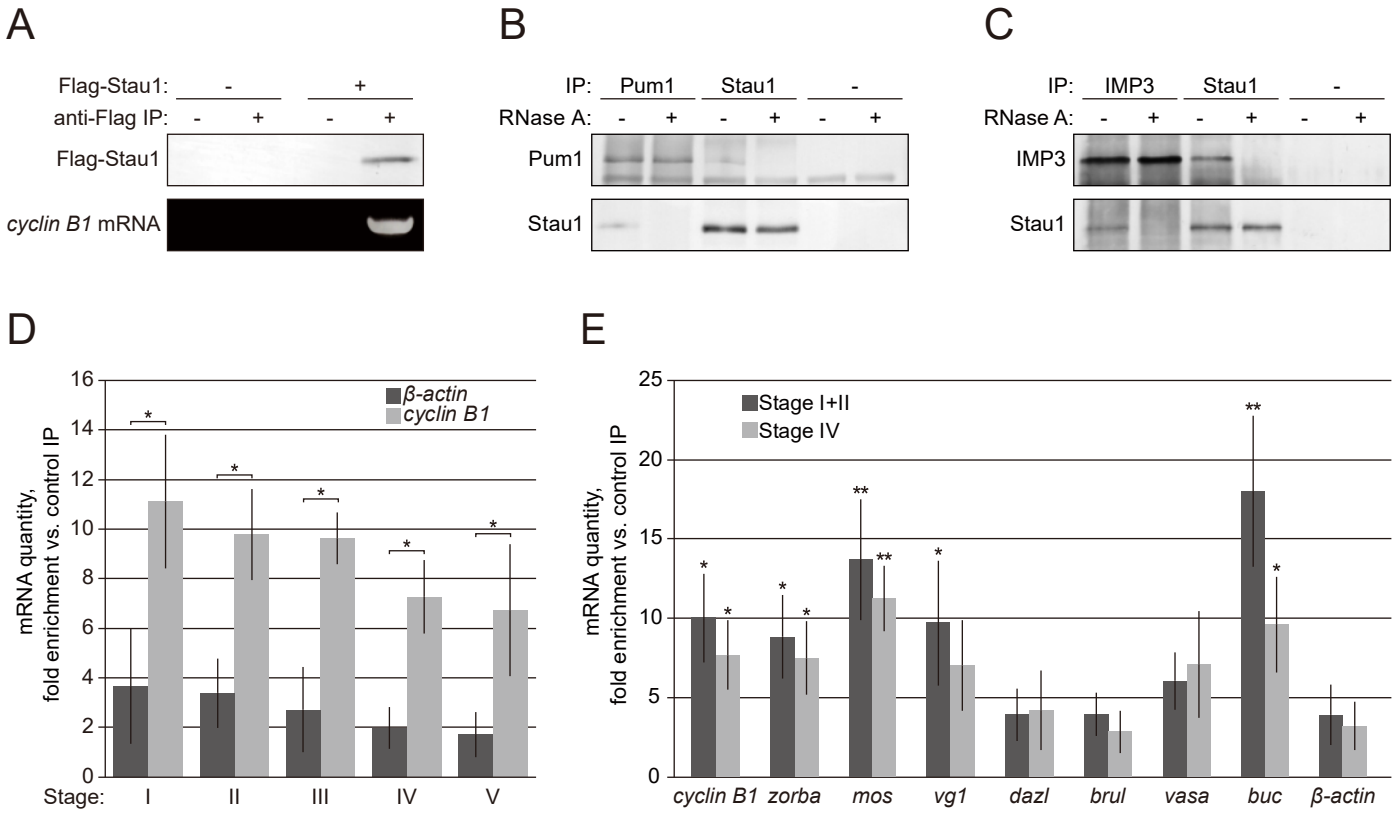

Fig. 8. Interaction of Stau1 with *cyclin B1* mRNA (A), Pumilio1 (Pum1, B), IMP3 (C) and mRNAs localized in zebrafish oocytes (D).

(A) Oocytes overexpressed with (+) or without (-) Flag-Stau1 were immunoprecipitated with (+) or without (-) anti-Flag antibody. Flag-Stau1 protein and *cyclin B1* mRNA in the immunoprecipitates were detected by immunoblotting with anti-Flag antibody and RT-PCR, respectively.

(B) Extracts from stage IV oocytes were treated with (+) or without (-) RNase A and immunoprecipitated with anti-Pum1, anti-Stau1 or control antibodies (-). Pum1 and Stau1 in the immunoprecipitates were detected by immunoblotting.

(C) Experiments similar to those in (B) using anti-IMP3 and anti-Stau1 antibodies.

(D) Immunoprecipitation followed by qRT-PCR analysis (IP/qRT-PCR). Extracts from stages I to V oocytes were immunoprecipitated with either anti-Stau1 antibody (anti-Stau1 IP) or control preimmune serum (control IP). Enrichment of *cyclin B1* mRNA and *β-actin* mRNA in anti-Stau1 IP relative to control IP was calculated. Values are means ± S.D. for three independent experiments. Statistical analyses were performed between *cyclin B1* mRNA and *β-actin* mRNA in each stage by two-tailed unpaired Student's *t*-test (*$P < 0.05$).

(E) Extracts from stages I and II oocytes or stage IV oocytes were subjected to IP/qRT-PCR using anti-Stau1 antibody (anti-Stau1 IP) or control preimmune serum (control IP) as in D. Enrichment of each mRNA in anti-Stau1 IP relative to control IP was calculated. Values are means ± S.D. for four independent experiments. Statistical analyses were performed between each mRNA and *β-actin* mRNA by two-tailed unpaired Student's *t*-test (*$P < 0.05$, **P <0.005).

Fig. 9

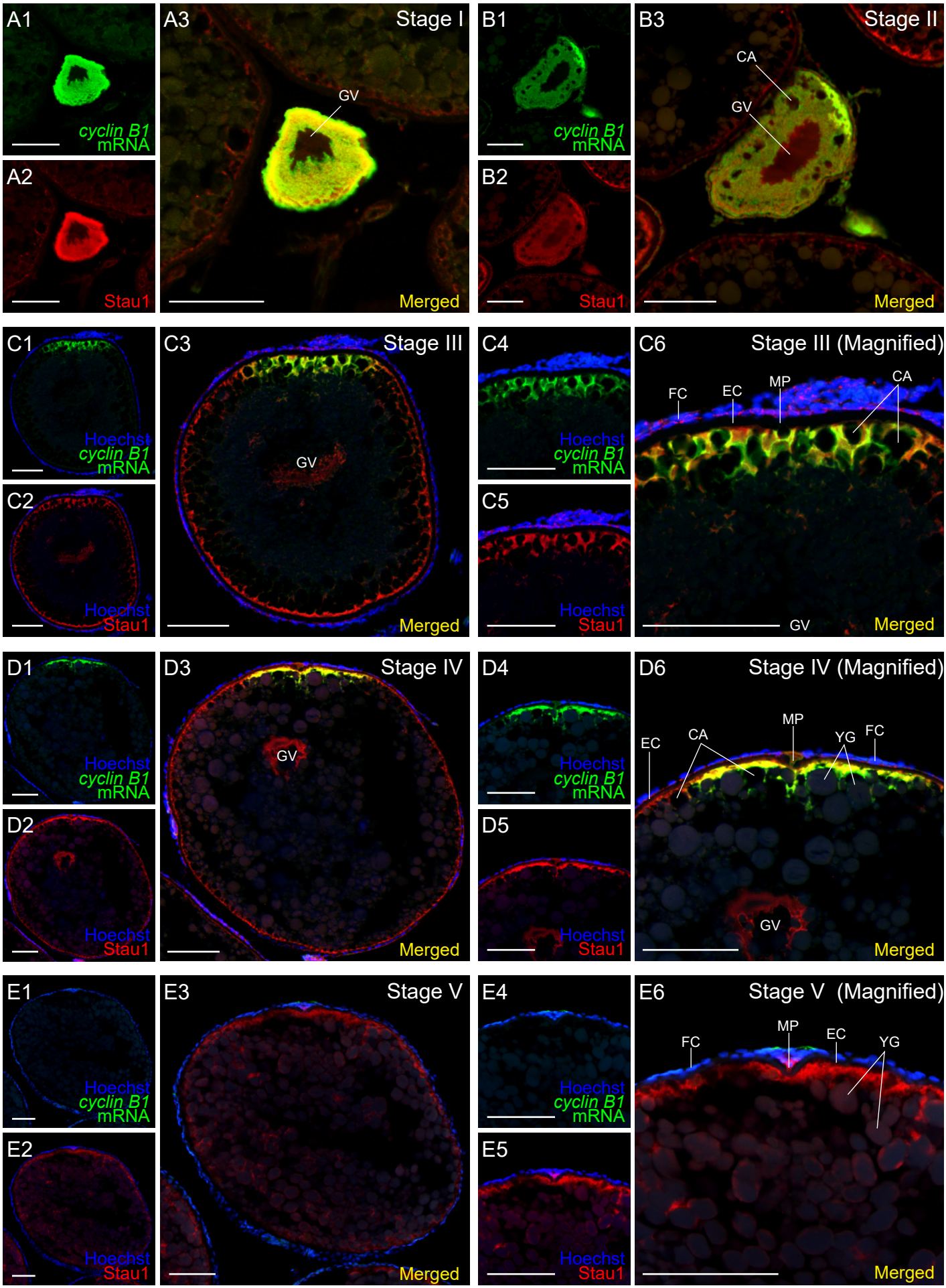
A1
cyclin B1 mRNA
A2
Stau1
A3
Stage I
GV
Merged
B1
cyclin B1 mRNA
B2
Stau1
B3
Stage II
CA
GV
Merged
C1
Hoechst
cyclin B1 mRNA
C2
Hoechst
Stau1
C3
Stage III
GV
Merged
C4
Hoechst
cyclin B1 mRNA
C5
Hoechst
Stau1
C6
Stage III (Magnified)
FC
EC
MP
CA
GV
Merged
D1
Hoechst
cyclin B1 mRNA
D2
Hoechst
Stau1
D3
Stage IV
GV
Merged
D4
Hoechst
cyclin B1 mRNA
D5
Hoechst
Stau1
D6
Stage IV (Magnified)
EC
CA
MP
YG
FC
GV
Merged
E1
Hoechst
cyclin B1 mRNA
E2
Hoechst
Stau1
E3
Stage V
Merged
E4
Hoechst
cyclin B1 mRNA
E5
Hoechst
Stau1
E6
Stage V (Magnified)
FC
MP
EC
YG
Merged

Fig. 9 (continued)

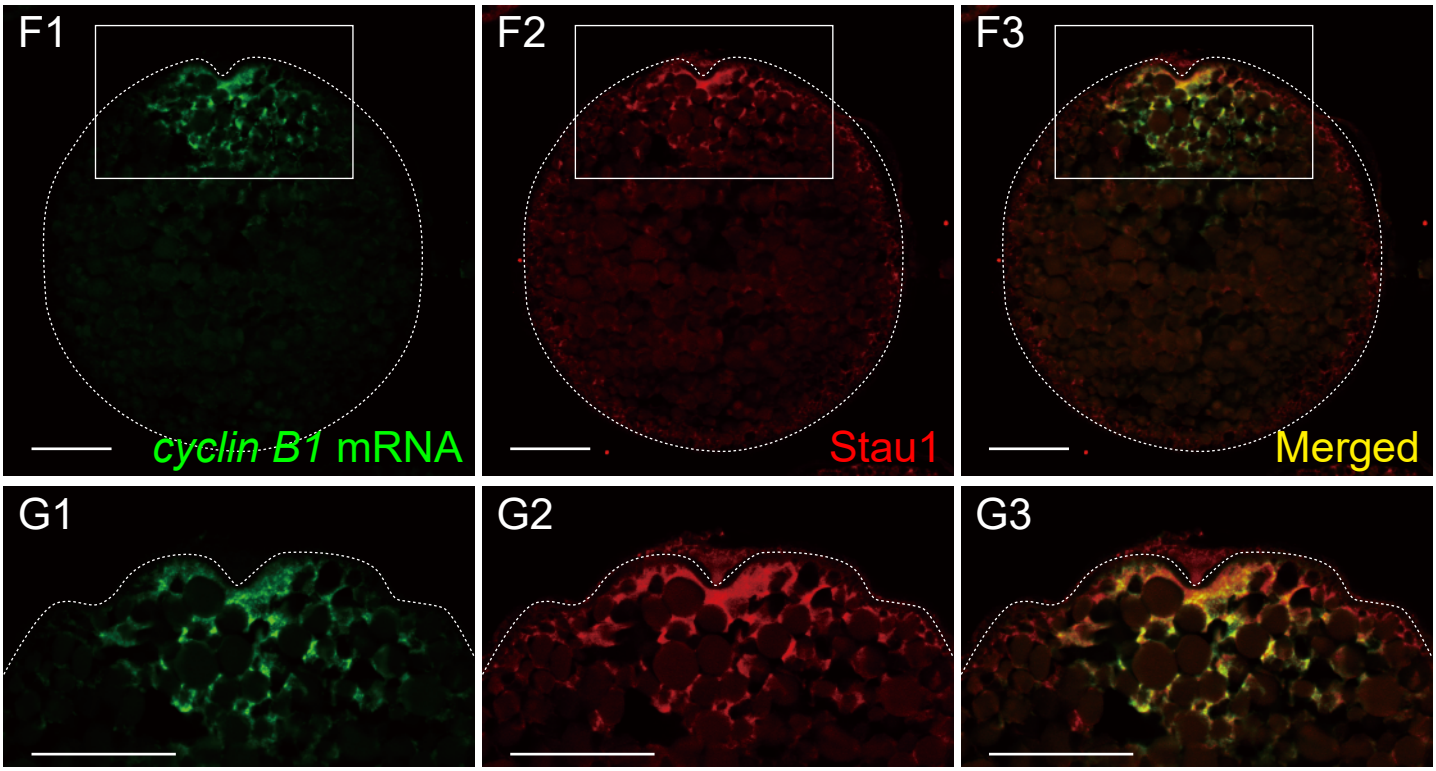

Fig. 9. Localization of *cyclin B1* mRNA and Stau1 protein in stage I (A), stage II (B), stage III (C), stage IV (D), stage V (E) and maturing (F and G) oocytes.

(A-E) Hoechst 33258 staining of DNA (blue: C-E), *in situ* hybridization of *cyclin B1* mRNA (green: A1; B1; C1, 4; D1, 4; E1, 4), immunostaining of Stau1 (red: A2; B2; C2, 5; D2, 5; E2, 5) and their merged and magnified images (A3; B3; C3, 6; D3, 6; E3, 6) are shown. GV, germinal vesicle; CA, cortical alveolus; EC, egg chorion; FC, follicle cell; MP, micropyle; YG, yolk granule. Scale bars, 100 μm.

(F and G) *In situ* hybridization of *cyclin B1* mRNA (green: F1; G1), immunostaining of Stau1 (red: F2; G2) and merged images (F3; G3). Magnified images of boxed regions in (F) are shown in (G). Oocytes are outlined by broken lines. Scale bars indicate 100 μm.

Fig. 10

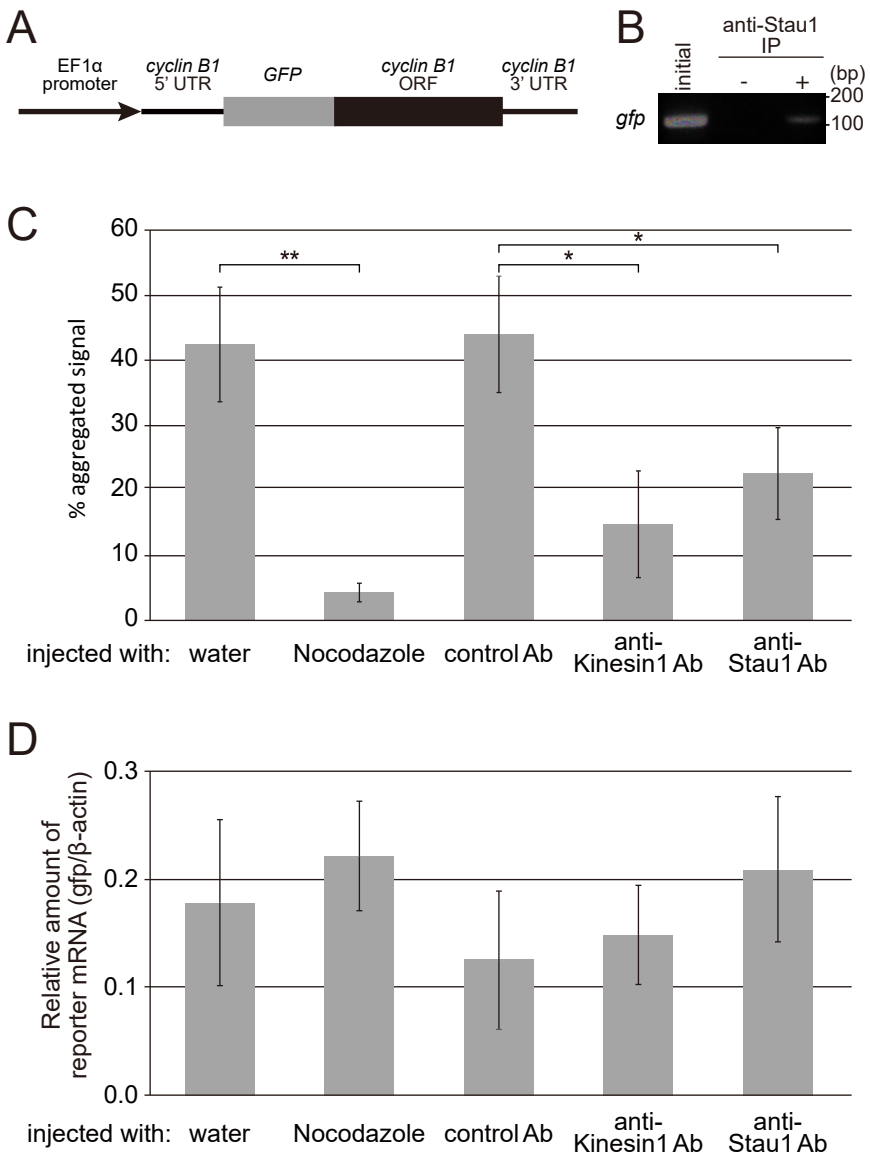

Fig. 10. Effects of a microtubule-depolymerizing drug (nocodazole) and antibodies against Kinesin1 and Stau1 on the aggregation of *gfp-cyclin* B1 reporter mRNA.

(A) Structure of the DNA construct to express *gfp-cyclin B1* reporter mRNA in oocytes. The construct was originally described as tgo3' in our previous report (Yasuda et al., 2010).

(B) Oocytes injected with reporter constructs were lysed and subjected to immunoprecipitation with (+) or without (-) anti-Stau1 antibody. The reporter mRNA in the immunoprecipitates (IP) and extracts before immunoprecipitation (initial) were detected by RT-PCR using the gfp sequence.

(C) Water, nocodazole, control anti-GST antibody, anti-Kinesin1 antibody or anti-Stau1 antibody was co-injected with the reporter vector into the nucleus and the cytoplasm of full-grown oocytes. Following whole-mount in situ hybridization, oocytes showing aggregated signals were counted. Values are means ± S.D. for three to five independent experiments. Statistical analysis was performed by two-tailed unpaired Student's *t*-test (**P* < 0.05, ***P* <0.005).

(D) Relative amounts of reporter mRNAs among injected oocytes in (D) normalized to those of *β-actin* mRNA. Values are means ± S.D. for three independent experiments.

Fig. 11

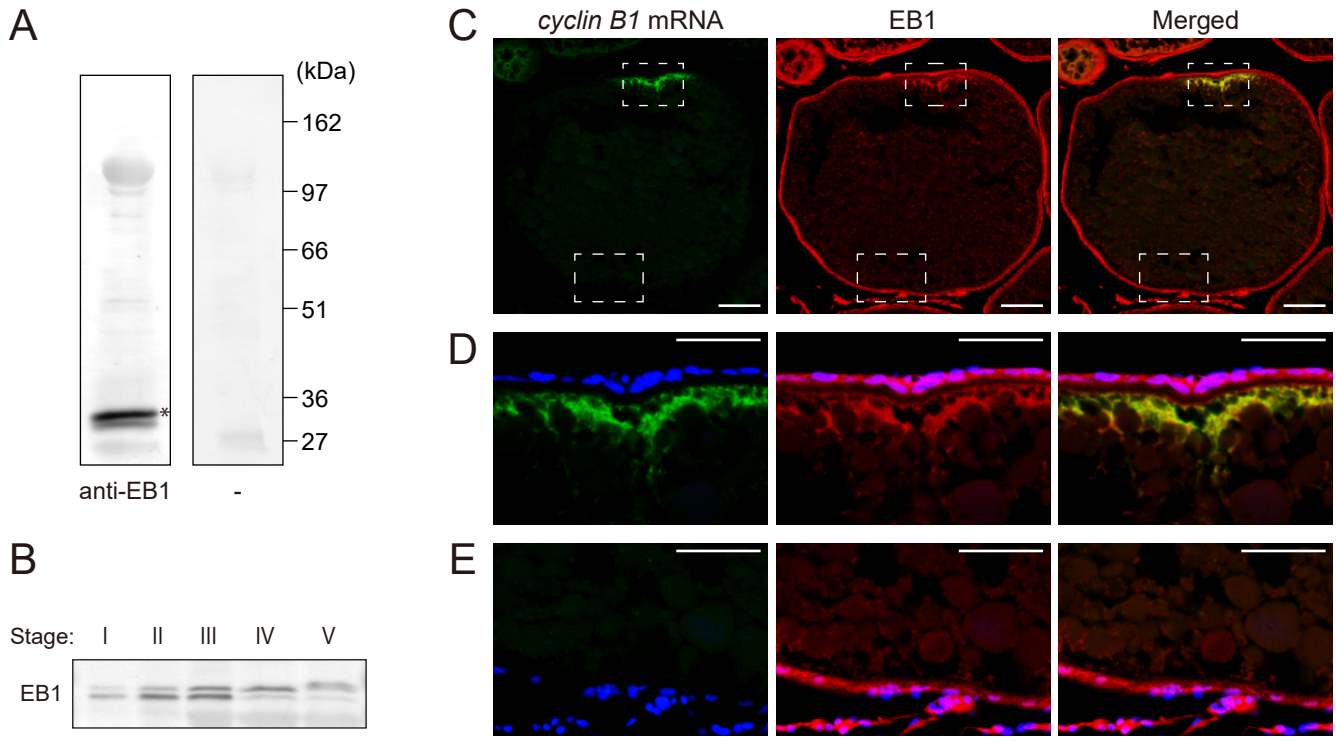

Fig. 11. Orientation of microtubules in zebrafish oocytes revealed by an antibody against EB1, a protein bound to the plus ends of microtubules.

(A) Crude extracts (10 μg) from stage IV oocytes were immunoblotted with anti-EB1 antibody or without the first antibody. Asterisk indicates EB1.

(B) Oocyte extracts equivalent to a single oocyte of stages I to V were analyzed by immunoblotting with anti-EB1 antibody.

(C) *In situ* hybridization of *cyclin B1* mRNA (green), immunostaining of EB1 (red) and merged images of stage IV oocytes. Scale bars indicate 100 μm.

(D and E) Magnified images of animal polar region (D) and vegetal polar region (E) shown in the boxed regions in (C). Hoechst 33258 staining of DNA is shown in blue. Scale bars indicate 50 μm.

Fig. 12

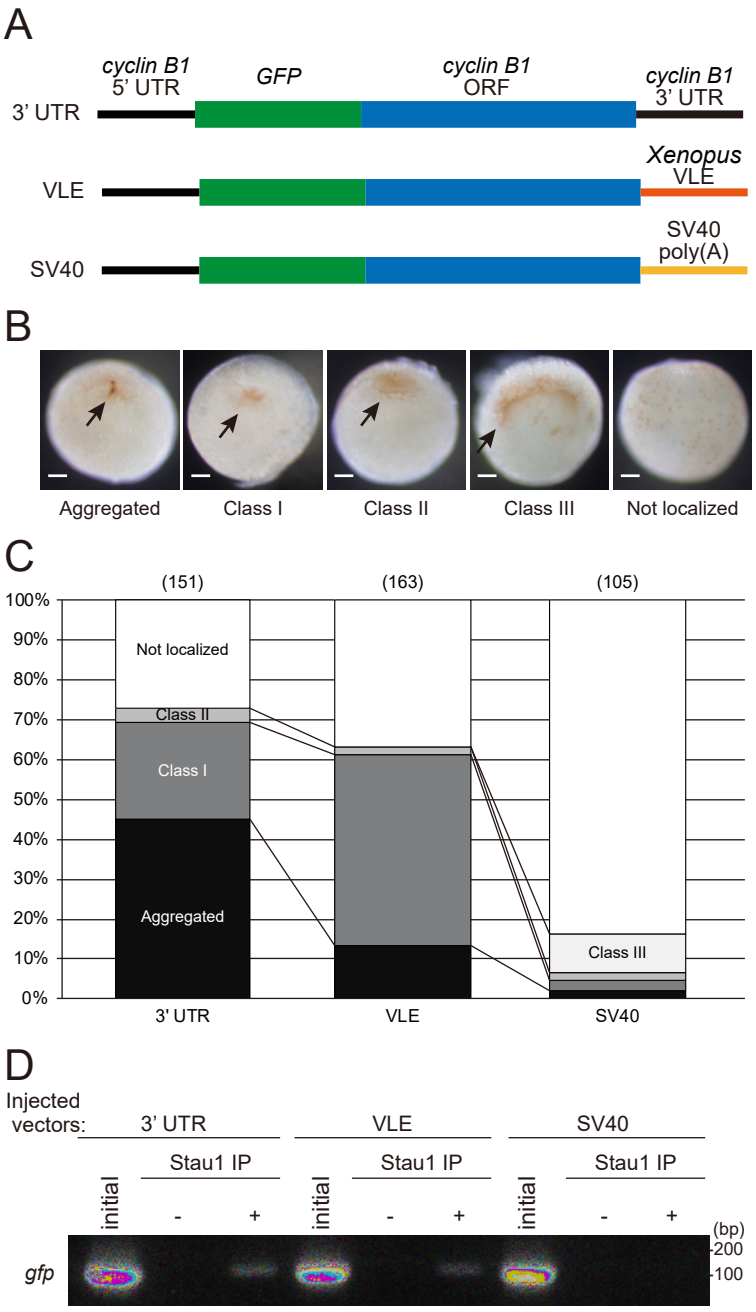

Fig. 12. Effects of 3' UTR sequences on the localization of *gfp-cyclin B1* mRNA reporter in zebrafish oocytes.

(A) Structures of reporter mRNAs. The *cyclin B1* 3' UTR sequence was replaced with either *Xenopus* vg1/vegetal localization element (VLE) or SV40 polyadenylation signal (SV40) sequences.

(B) Localized reporter mRNAs were detected by whole-mount *in situ* hybridization using a gfp probe, and the resulting signals were classified into 5 groups. Arrows indicate localized signals. Scale bars indicate 100 μm.

(C) The numbers of oocytes showing each localized signal were counted. Data were collected from three independent experiments. The numbers in parentheses indicate the total numbers of oocytes analyzed.

(D) Extracts from oocytes injected with reporter constructs (3' UTR, VLE, SV40) were subjected to immunoprecipitation with (+) or without (-) anti-Stau1 antibody (Stau1 IP). The reporter mRNAs in the immunoprecipitates (IP) and extracts before immunoprecipitation (initial) were detected by RT-PCR using the gfp sequence.

Fig. 13

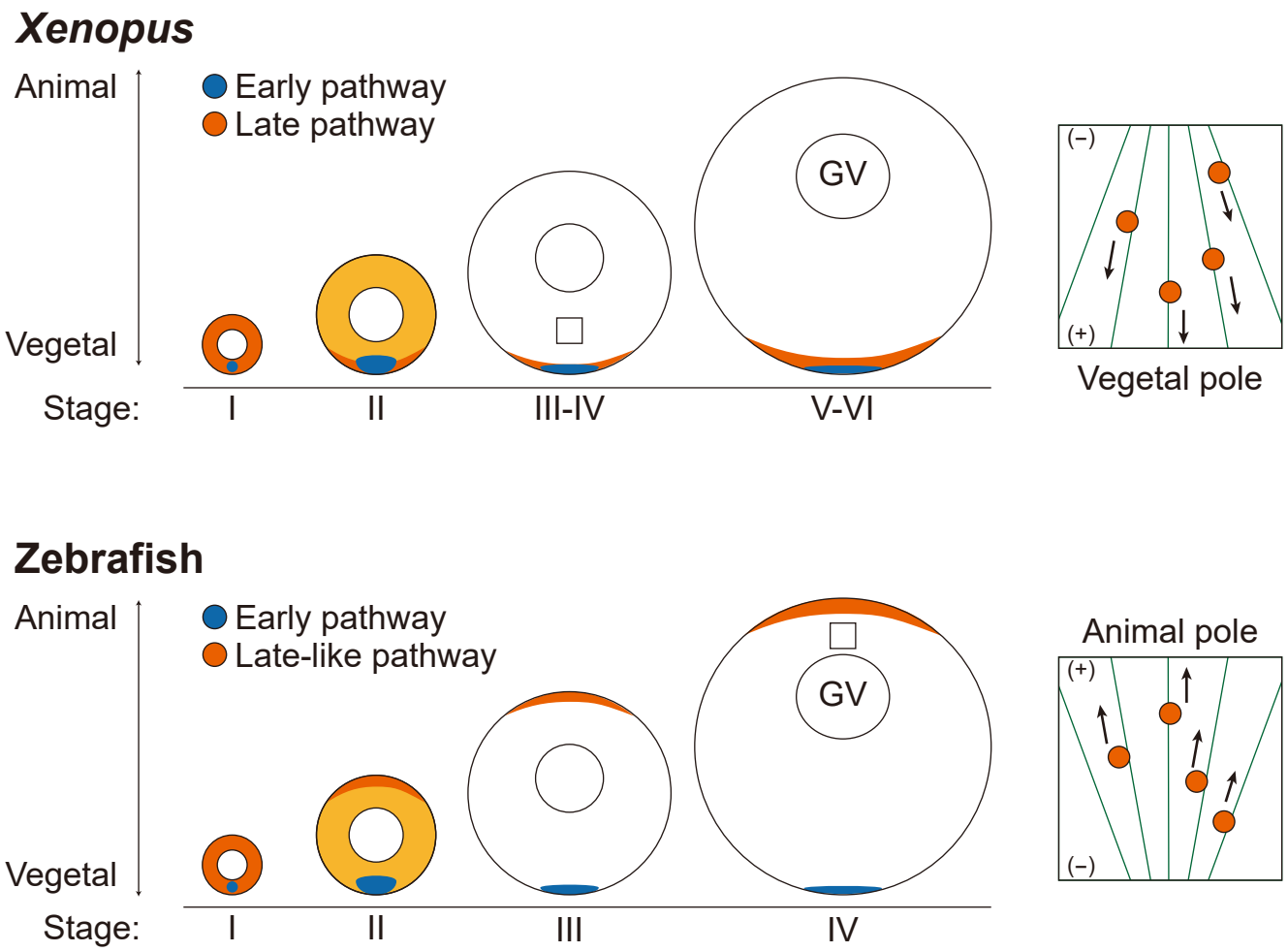

Fig. 13. Generality and diversity in the mRNA localization machinery in *Xenopus* and zebrafish oocytes. The early (METRO) pathway is shown in blue, and the late (vg1) pathway and late-like pathway are shown in orange. Boxed regions in the oocytes are magnified on the right. Green lines and (+)/(-) indicate microtubules and their polarities. Red circles and arrows indicate mRNA-protein complexes moving toward the plus ends of microtubules. Comparison of the oocyte stages in *Xenopus* (Dumont, 1972) and zebrafish (Selman et al., 1993) is cited from the previous study (Houston, 2013). Not to scale.

Fig. 14

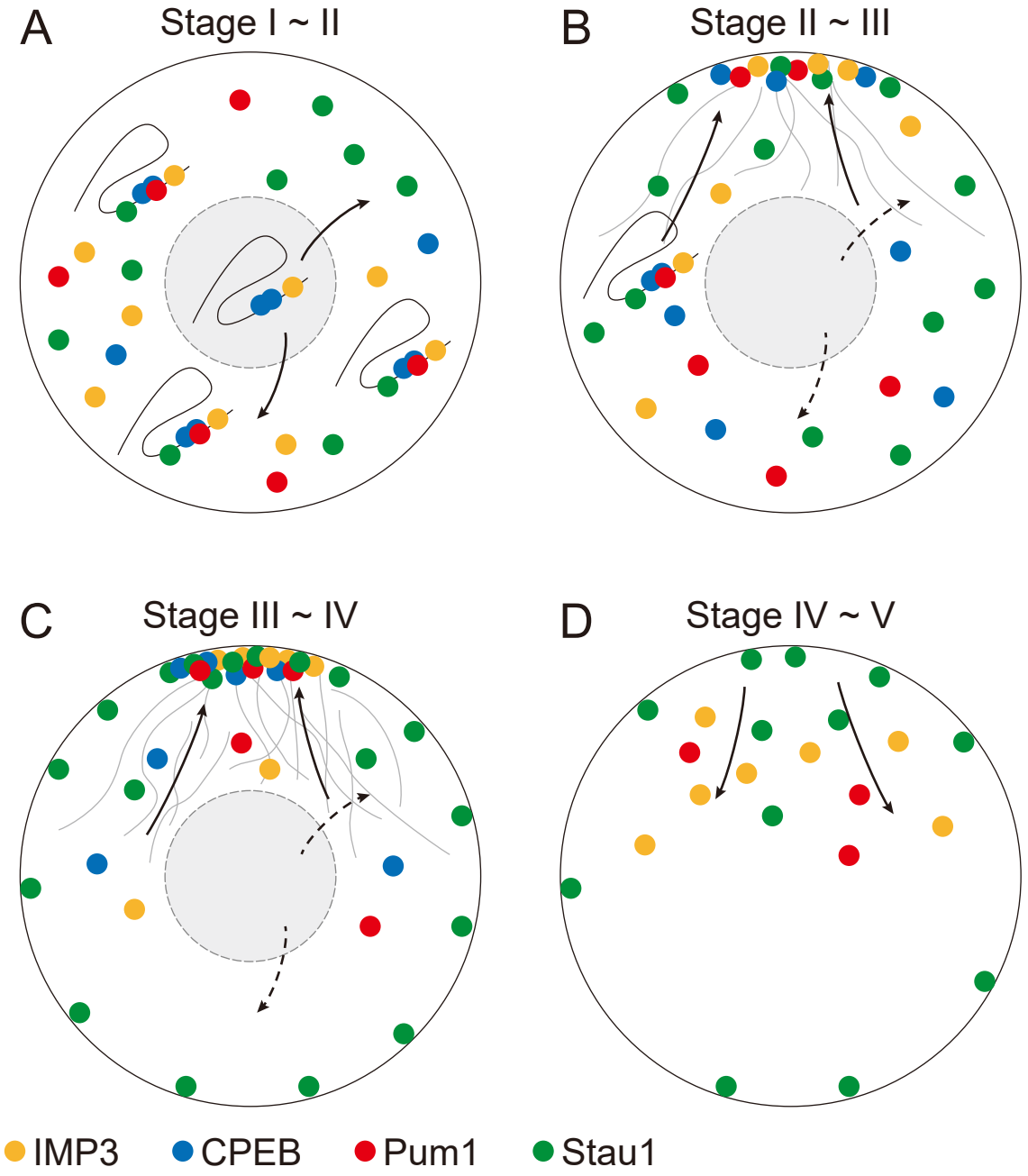

Fig. 14. Model for the post-transcriptional regulation of *cyclin B1* mRNA during oocyte growth and maturation.

Behavior of *cyclin B1* mRNA (black lines, not shown in C and D) in oocytes of stages I to II (A), II to III (B), III to IV (C) and IV to V (D). Colored dots represent IMP3 (orange), CPEB (blue), Pum1 (red) and Stau1 (green). Arrows indicate the movements of *cyclin B1* mRNA. Microtubules are shown by gray lines and GVs are shown by the gray circles. Animal pole upward. Not to scale.