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A *cis*-acting element in the coding region of *cyclin B1* mRNA couples subcellular localization to translational timing

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Running title: Novel *cis*-element in *cyclin B1* mRNA

Abstract

Subcellular localization of messenger RNAs (mRNAs) to correct sites and translational activation at appropriate timings are crucial for normal progression of various biological events. However, a molecular link between the spatial regulation and temporal regulation remains unresolved. In immature zebrafish oocytes, translationally repressed *cyclin B1* mRNA is localized to the animal polar cytoplasm and its temporally regulated translational activation in response to a maturation-inducing hormone is essential to promote oocyte maturation. We previously reported that the coding region of *cyclin B1* mRNA is required for the spatio-temporal regulation. Here, we report that a sequence, CAGGAGACC, that is conserved in the coding region of vertebrate *cyclin B1* mRNA is involved in the regulation. Like endogenous *cyclin B1* mRNA, reporter mRNAs harboring the sequence CAGGAGACC were localized to the animal polar cytoplasm of oocytes, while those carrying mutations in the sequence (with no change in the coding amino acids) were dispersed in the animal hemisphere of oocytes. Furthermore, translational activation of the mutant mRNAs was initiated at a timing earlier than that of endogenous and wild-type reporter mRNAs during oocyte maturation. Interaction of CAGGAGACC with proteins *in vitro* suggests that this sequence functions in collaboration with a *trans*-acting protein factor(s) in oocytes. These findings reveal that the sequence in the coding region of *cyclin B1* mRNA plays an important role as a *cis*-acting element in both subcellular localization and translational timing of mRNA, providing a direct molecular link between the spatial and temporal regulation of mRNA translation.

Key words: RNA localization, cyclin B1, oocyte maturation, translational control

Introduction

Localization and translational regulation of messenger RNAs (mRNAs) have emerged as a common and important mechanism for the control of protein production at restricted regions and appropriate timings in various biological processes, including cell differentiation, body patterning and synaptic plasticity in a wide range of organisms (Martin 2004; Du et al. 2007; Holt and Bullock 2009). The spatio-temporal regulation of mRNA can be classified into two types, spatial regulation (short-term repression) and temporal regulation (long-term repression). In spatial regulation, mRNAs are transported from the nucleus to a specific site in the cytoplasm, during which time mRNAs are translationally repressed, but they are translated promptly when they have reached their final destination without any apparent stimuli. In this case, no time lag exists between the timing at which mRNA reaches the final destination and the timing at which translation of mRNA is initiated. In the temporal regulation, mRNAs remain repressed even after reaching their final destination and they are translationally activated only when the cell is stimulated by valid signals. In this case, the timing of mRNA translation would be delayed relative to the timing of mRNA settlement to its final destination. Both types of translational regulation rely on *cis*-acting elements usually located in the 3' untranslated region (UTR) and *trans*-acting factors bound specifically to the *cis*-elements (Martin and Ephrussi 2009). Molecular mechanisms of the spatial regulation have been investigated using yeast and mammalian cells. It has been shown that *trans*-acting RNA-binding proteins, such as She2p in yeast and ZBP1 in mammalian cells, control both mRNA localization and translational repression,

demonstrating a direct link between the two events (Hüttelmaier et al. 2005; Du et al. 2008; Shen et al. 2009). In contrast, mechanisms underlying temporal regulation are largely unknown. Indeed, it remains to be elucidated what mechanism couples the localization of mRNA with the timing of translational activation.

Oogenesis and oocyte maturation are typical processes in which temporal regulation of mRNA translation operates. During oogenesis, translationally repressed mRNAs are stored in the oocyte cytoplasm. In response to a maturation-inducing hormone (MIH), full-grown oocytes undergo oocyte maturation, during which time mRNAs stored in oocytes, such as *mos*, *cyclin B1* and *wee1*, are translationally activated at a timing specific to each mRNA (Charlesworth et al. 2000; see also review, Mendez and Richter 2001; de Moor et al. 2005). The translational activation of *cyclin B1* mRNAs is well known to play an important role in initiating oocyte maturation and assuring meiotic metaphase I to II transition without DNA replication (Kondo et al. 1997; Ihara et al. 1998; Hochegger et al. 2001; Ledan et al. 2001; Kotani and Yamashita 2002; Haccard and Jessus 2006; Gaffré et al. 2011). In immature zebrafish oocytes, translationally repressed *cyclin B1* mRNAs are localized to the animal polar cytoplasm as an aggregation (Kondo et al. 2001; Yasuda et al. 2010). When oocytes are stimulated with an MIH, the aggregated *cyclin B1* mRNAs are dispersed and translationally activated to synthesize Cyclin B1 proteins, which, in turn, bind directly to preexisting Cdc2 proteins to form a maturation-promoting factor (MPF), the final trigger of oocyte maturation (Kondo et al. 1997; Kondo et al. 2001; see also review, Nagahama and Yamashita 2008).

To analyze molecular and cellular mechanisms of temporally regulated translation of *cyclin B1* mRNA, we developed a genetic experimental system in zebrafish, in which reporter mRNAs are transcribed in the nucleus from transgenes (Yasuda et al. 2010). Translational activation of reporter mRNAs can be visualized in real time by a fluorescence dye, ReAsH, which emits fluorescence immediately after binding to a nascent tetra cysteine (TC) tag encoded by reporter mRNAs. This experimental system revealed that the coding region of *cyclin B1* mRNA is involved in both correct anchoring (aggregation) of mRNA at the animal polar cytoplasm and in translational activation at the accurate timing after MIH stimulation, although the presence of a 3' UTR is a prerequisite for mRNA transport to the animal hemisphere and for translational control (Yasuda et al. 2010). However, it is still not known whether the mRNA anchoring and translational timing are regulated by different elements or by a single element existing in the coding region and what molecules link the two mechanisms.

In this study, we found a novel *cis*-acting element consisting of 9 nucleotides (nts), CAGGAGACC, in the coding region of *cyclin B1* mRNA by examining the localization and translational timings of reporter mRNAs carrying deletions in the coding region or mutations in this sequence. The 9-nt sequence is required for *cyclin B1* mRNA to be anchored at the animal polar cytoplasm and activated at the accurate timing during oocyte maturation. An electrophoretic mobility shift assay showed that the 9-nt sequence interacts with proteins *in vitro*. These findings suggest that, in cooperation with a *trans*-acting protein(s), the 9-nt sequence dictates both the correct anchoring

(aggregation) and accurate timing of translation of *cyclin B1* mRNA, providing a direct link between the localization and temporal regulation of translation.

Materials and methods

Construction of reporter genes

Fragments of the coding sequence of zebrafish *cyclin B1* were amplified by PCR with a full-length zebrafish *cyclin B1* cDNA (Kondo et al. 1997) and the following primers (underlines indicating *Bst*BI sites): 1-523 with cycB1-ORF-f1 (5′-TTC GAA ATG ATG GCT CTC CGG TGT C-3′) and cycB1-region1-R (5’-TTC GAA AGT CGA TAA GAA TGG CAC GC-3’); 524-1197 with cycB1-region2-F (5′-TTC GAA GGC TTG TGC AAG TCC AGA TT-3′) and cycB1-ORF-r1 (5′-TTC GAA CTA TGA GGA TCT GCT TAG CC-3′); 524-736 with cycB1-region2-F (5′-TTC GAA GGC TTG TGC AAG TCC AGA TT-3′) and cycB1-region3-R (5′-TTC GAA TGG TGT ATG CAC GGT CTG TC-3′); 737-949 with cycB1-region4-F (5′-TTC GAA CCA GTC AGA TCC GGG AGA TG-3′) and cycB1-region4-R (5′-TTC GAA CAT AAG CAG CAC TGG CCA TC-3′); 949-1197 with cycB1-region5-F (5′-TTC GAA CCC TGA CCC TGA AGG TCT TC-3′) and cycB1-ORF-r1 (5′-TTC GAA CTA TGA GGA TCT GCT TAG CC-3′). The PCR products were inserted into the *Cla*I site of the *tg3’* construct (Yasuda et al. 2010). The *tgo^{M}3’* construct was produced using a QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) with a primer set of ORF-mut-F (5’-GTT TAG GCT TCA AGA AAC GAT GTA CAT GAC TGT TGC C-3’) and ORF-mut-R (5’-GGC AAC AGT CAT GTA CAT CGT TTC TTG AAG CAG CCT AAA C-3’) and the *tgo3′* construct

(Yasuda et al. 2010) as a template. Similarly, the *tgcis-3'* construct was produced with a primer set of GFP-cis-F (5'-GGACGAGCTGTACAAGCAGGAGACCTAAAGCGGCCGCCACCGCGG -3') and GFP-cis-R (5'-CCGCGGTGGCGGCCGCTTTAGGTCTCCTGCTTGTACAGCTCGTCC-3') and the *tg3'* construct as a template. The SV40 reporter gene was produced by inserting the full-length *cyclin B1* ORF into the *tgs* construct.

Preparation of oocytes

All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University (permission No. 08-0013). Oocytes were manually isolated from ovaries with a pair of fine forceps in zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES; pH 7.2) under a dissecting microscope.

Injection of reporter constructs into the nucleus of zebrafish oocytes

Manually isolated full-grown oocytes were used to inject 25 pg of reporter constructs into the nucleus using a microinjector, CellTram vario (Eppendorf), as reported previously (Kress et al. 2004; Lin et al. 2010). After injection, oocytes were incubated in zebrafish Ringer's solution for 4 hours. The oocytes were fixed with 4% paraformaldehyde in phosphate buffered saline (4% PFA/PBS) (PBS: 137 mM NaCl,

2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4; pH 7.2) overnight at 4°C and used for whole-mount *in situ* hybridization.

Production of transgenic zebrafish

Transgenic zebrafish were produced by using *Tol2* transposon-mediated germline transmission (Kotani et al. 2006). One nanoliter of a solution containing 250 µg/ml of reporter constructs and 250 µg/ml of *in vitro*-synthesized mRNAs encoding the *Tol2* transposase was injected into one-cell stage embryos. The injected embryos were raised and crossed with wild-type fish. F1 embryos were screened by GFP fluorescence under an M165FC fluorescence stereomicroscope (Leica). The embryos expressing GFP were raised to adulthood and the ovaries from F1 to F8 females were used for whole-mount and section *in situ* hybridization analyses and real-time imaging.

Whole-mount and section in situ hybridization analyses

Digoxigenin (DIG)-labeled anti-sense probes for the *gfp* coding region and full-length *cyclin B1*, *cyclin B2* and *cyclin A1* coding regions were synthesized with DIG RNA labeling mix (Roche) according to the manufacturer's instructions. Whole-mount *in situ* hybridization analysis of oocytes injected with reporter constructs or derived from transgenic zebrafish was performed according to the procedure reported previously (Schulte-Merker et al. 1992). Section *in situ* hybridization analysis of ovaries was performed according to the procedure reported previously (Kondo et al. 2001).

Real-time PCR

Reporter mRNA expression in full-grown oocytes was quantified by using a real-time PCR system with SYBR green PCR Master Mix (Applied Biosystems) as described previously (Yasuda et al. 2010).

Real-time imaging

To detect translation of reporter mRNAs in oocytes, 1 nl of 0.2 mM ReAsH in zebrafish Ringer's solution was injected into full-grown oocytes using the CellTram vario. Oocyte maturation was induced by stimulation with 17α,20β-dihydroxy-4-pregnen-3-one (final concentration, 1 μg/ml), an MIH in fish. The ReAsH fluorescent signal was observed under an M165FC fluorescent stereomicroscope (Leica) using a filter set of Texas Red and photographed by using a VB-7010 cooled CCD color camera (Keyence) at intervals of 15 min after MIH stimulation.

Jasplakinolide treatment

Manually isolated oocytes were incubated with 5 μM jasplakinolide or 0.5% DMSO in zebrafish Ringer's solution at room temperature for 140 min. Half of them were simultaneously stimulated with MIH. For detection of *cyclin B1* and *tgo3'* mRNAs, the oocytes were fixed 140 min after MIH and jasplakinolide treatment and analyzed by section *in situ* hybridization. For real-time detection of translation of *tgo3'* and *tgo^{M}3'* mRNAs, 1 nl of 0.2 mM ReAsH was injected into full-grown oocytes using the

microinjector CellTram vario. The oocytes were incubated with jasplakinolide and MIH, observed under an M165FC fluorescent stereomicroscope, and photographed by using a VB-7010 cooled CCD color camera. For detection of Cdc2 and Cyclin B1 by immunoblotting, the oocytes were homogenized with ice-cold extraction buffer (100 mM β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 300 µM PMSF, 3 µg/ml leupeptin; pH 7.5). Extracts were precipitated with Suc1 beads. The samples were separated by SDS-PAGE, blotted onto an Immobilon membrane, and probed with anti-Cdc2 (MC2-21) (Tanaka and Yamashita 1995) and anti-goldfish Cyclin B1 (B112) (Katsu et al. 1993) monoclonal antibodies.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (Shcherbakov and Piendl 2007; Zamore et al. 1997). Briefly, 50 oocytes were extracted with 50 µl of binding buffer 1 (20 mM HEPES-KOH; pH 7.4, 100 mM KCl, 0.1 mM EDTA, 0.02% (w/v) Tween-20 and 0.1 mg/ml BSA) containing protease inhibitors [1 mM dithiothreitol (DTT), 300 µM (p-amidinophenyl) methanesulfonyl fluoride (PMSF), 30 µg/ml leupeptin]. Using total RNAs extracted from zebrafish, frog (*Xenopus laevis*) or mouse oocytes, cDNA fragments including 524-574 nts of zebrafish *cyclin B1*, 567-617 nts of frog *cyclin B1* and 599-647 nts of mouse *cyclin B1* were amplified by RT-PCR and cloned into pGEM T-easy vector (Promega). RNA probes were synthesized with T7 RNA polymerase (Invitrogen) and diluted in distilled water. The RNA probes (250 ng per reaction) were incubated with the oocyte extracts diluted in binding buffer 2 (1 mM EDTA, 10 mM

HEPES; pH 7.4, 50 mM KCl, 2 mM DTT, 0.5 unit of RNAsin plus (Promega), 0.1 mg/ml BSA and 0.01% (w/v) Tween-20) to 20 µl of total reaction volume at room temperature for 20 minutes. After added loading buffer of the EMSA kit (Molecular probes), the samples were loaded in 6% non-denaturing polyacrylamide gel under a constant current condition (16 mA/gel) for 30-50 minutes. The RNA probes were stained with SYBR green EMSA nucleic acid gel stain (Molecular probes) according to the manufacturer's instructions. SYBR green fluorescence was detected by using LAS-3000 (Fujifilm).

Results

Sequences in the cyclin B1 coding region responsible for mRNA aggregation

In addition to the 3'UTR of *cyclin B1* mRNA, the coding region is crucial for the correct subcellular localization of mRNA (Yasuda et al. 2010). To identify sequences involved in this mRNA localization, we constructed reporter genes that contain variously truncated coding regions of *cyclin B1* (Fig. 1A). Since the production of transgenic zebrafish, in which reporter mRNAs are stably transcribed in oocytes, is time-consuming, we used an easy way as a first step toward identification of the region involved in mRNA localization. We previously reported that mRNAs transcribed in the nucleus from transgenes recapitulate the localization of endogenous mRNAs, although those synthesized *in vitro* and injected directly into the oocyte cytoplasm do not (Yasuda et al. 2010). We therefore injected reporter genes into the oocyte nucleus and analyzed the localization of transcribed mRNAs by whole-mount *in situ* hybridization with a *gfp*

probe. Endogenous *cyclin B1* mRNAs are localized as an aggregation to a space less than 200 μm in diameter at the animal polar cytoplasm beneath the micro-pile, a single opening of the egg chorion through which the fertilizing spermatozoon enters the oocytes (Figs. 2A, 4A). Since the extents of mRNA localization in the oocytes varied in reporter mRNAs, signals less than 200 μm in diameter were defined as 'aggregation' and those wider than 200 μm were defined as 'dispersion'.

Oocytes injected with distilled water showed no signals, while oocytes injected with a reporter gene containing a full-length *cyclin B1* (referred to as *tgo3'*; Yasuda et al. 2010) exhibited aggregated signals in the animal polar cytoplasm (Fig. 2B, C; Table 1), confirming that the reporter mRNAs transcribed in the nucleus recapitulate the behavior of endogenous mRNAs. Reporter mRNAs containing 524-1197 nts were aggregated in the cytoplasm at the animal pole as in the case of *tgo3'*, whereas those containing 1-523 nts were dispersed widely in the animal hemisphere of oocytes (Fig. 2D, E; Table 1). These results indicate that 524-1197 nts contain an element(s) responsible for the correct localization (anchoring at the animal polar cytoplasm as an aggregation) of *cyclin B1* mRNA. We then divided 524-1197 nts into three fragments. Reporter mRNAs containing 524-736 nts were aggregated, whereas those containing 737-949 or 949-1197 nts were not (Fig. 2F-H; Table 1). Therefore, 524-736 nts in the *cyclin B1* coding region are required for the anchoring.

Results of experiments in which reporter genes were injected into the nucleus revealed that 524-736 nts in the *cyclin B1* coding region are required for correct anchoring of *cyclin B1* mRNA at the animal polar cytoplasm. We confirmed this result

by producing transgenic zebrafish that stably transcribe reporter mRNAs in oocytes (Yasuda et al. 2010). We produced transgenic lines carrying reporter genes containing the full length, 1-523, 524-1197, 524-736, 737-949 or 949-1197 nts of the *cyclin B1* coding region by *Tol2* transposon-mediated germ-line transmission (Kotani et al. 2006). To avoid the effects of sites in the genome to which the transgenes were inserted, we analyzed more than two transgenic lines carrying the same reporter gene by whole-mount (Fig. 3) and section (Fig. 4) *in situ* hybridizations and confirmed that the same reporter gene provided equivalent results. Quantification of reporter mRNAs by real-time PCR showed no statistical differences (Fig. 3K). Consistent with the results of injection experiments, reporter mRNAs containing full length, 524-1197 or 524-736 nts were aggregated (Fig. 3B, D, E; Table 2) in the animal polar cytoplasm (Fig. 4B, D, E), as in the case of endogenous *cyclin B1* mRNAs (Figs. 3A, 4A). In contrast, reporter mRNAs containing 1-523, 737-949 or 949-1197 nts were shown to be dispersed throughout the animal hemisphere of oocytes by whole-mount *in situ* hybridization (Fig. 3C, F, G; Table 2), and clear signals were not found in the cytoplasm beneath the micro-pile by section *in situ* hybridization (Fig. 4C, F, G), probably because the concentration of reporter mRNAs decreased to undetectable levels by dispersion in the cytoplasm. In agreement with our previous finding that a reporter mRNA, *tgs*, lacking *cyclin B1* 3' UTR shows no localization (Yasuda et al. 2010), a reporter mRNA that contains SV40 3' UTR instead of *cyclin B1* 3' UTR (Fig. 3J; cf. *tgo3'* in Fig. 1A) showed no aggregation (Fig. 3I), indicating that the presence of *cyclin B1* 3' UTR is a prerequisite for mRNA localization. We therefore concluded that the 524-736 sequence

of the *cyclin B1* coding region is responsible for anchoring (correct localization) of the mRNA in the presence of the 3' UTR.

Requirement of 524-736 nts of the cyclin B1 coding region for translational activation at accurate timing during oocyte maturation

We previously reported that, although translational repression of *cyclin B1* mRNA is primarily dependent on the 3'UTR, accurate timing of translational activation after induction of oocyte maturation requires the coding region (Yasuda et al. 2010). To identify the sequence responsible for the temporal regulation of mRNA translation, we analyzed the timings of translational activation of reporter mRNAs by real-time imaging (Yasuda et al. 2010). Since oocytes derived from different females undergo germinal vesicle breakdown (GVBD) at different timings after induction of oocyte maturation (60 to 150 min), the time when the oocytes initiate GVBD after MIH stimulation is expressed as $T_{GVBD} = 100$, and the timing of translation is expressed relative to this time.

The timings of translational activation of reporter mRNAs containing the full length (*tgo3'*), 524-1197 or 524-736 nts of the *cyclin B1* coding region were similar (T_{GVBD} = 51.3 ± 2.6 for *tgo3'*, 46.0 ± 2.1 for 524-1197, and 54.6 ± 8.6 for 524-736) (Fig. 5A, C, D, H). In contrast, reporter mRNAs containing 1-523, 737-949 or 949-1197 nts were translated at a significantly earlier timing (T_{GVBD} = 21.0 ± 3.0 for 1-523, 25.8 ± 3.2 for 737-949, and 18.0 ± 3.3 for 949-1197) (Fig. 5B, E, F, H). Therefore, the 524-736 sequence that is responsible for the anchoring of *cyclin B1* mRNA (Figs. 2-4) is also

involved in translational timing during oocyte maturation.

Evolutionally conserved sequence responsible for both anchoring and translational timing of cyclin B1 mRNA

Since *cyclin B1* mRNAs exhibit subcellular localization in immature oocytes of zebrafish and frogs (Kondo et al. 2001; Groisman et al. 2000; Yausda et al. 2010), we predicted that a sequence responsible for mRNA localization might be evolutionally conserved. Alignment of 524-736 nts of the zebrafish *cyclin B1* coding region with corresponding sequences of human, mouse, and frog *cyclin B1* enabled identification of a conserved sequence, CAGGAGACC, at 559-567 nts of the zebrafish coding region (Fig. 1B). This sequence encodes amino acids of Gln, Glu and Thr (Fig. 1C). Despite the fact that the third nucleotide of each codon can be replaced with an A in the first and second codons and with an A, G or U in the third codon, all of the third nucleotides are completely conserved, suggesting that this sequence not only encodes evolutionally conserved amino acids but also provides a basis for translational regulation through the RNA sequence itself.

To determine whether the 9-nt sequence functions as a *cis*-acting element in mRNA anchoring and translational timing regulation, we constructed a reporter gene containing a full-length *cyclin B1* with mutations in the sequence CAGGAGACC (CAAGAAACG, underlines indicating mutated sites), which encodes amino acids identical to those encoded by the original sequence (referred to as *tgo^{M}3'*, Fig. 1A). We first injected the *tgo^{M}3'* construct into the nucleus of zebrafish oocytes and examined mRNA localization

by whole-mount *in situ* hybridization. The *tgo^{M}3'* reporter mRNAs were dispersed in the cytoplasm of the animal hemisphere (Fig. 2I; Table 1), suggesting a function of CAGGAGACC in mRNA anchoring. We then produced transgenic zebrafish carrying this reporter gene and examined the localization and translational timing of reporter mRNAs in oocytes. The *tgo^{M}3'* mRNAs were not aggregated in the animal polar cytoplasm beneath the micro-pile (Fig. 4H) but were widely distributed in the animal hemisphere of oocytes (Fig. 3H; Table 2). Furthermore, the *tgo^{M}3'* mRNAs were translated at a timing earlier than that of *tgo3'* mRNAs after MIH stimulation (T_{GVBD} = 23.3 ± 1.2 for *tgo^{M}3'*, 51.3 ± 2.6 for *tgo3'*) (Fig. 5G, H). These results indicate that the sequence CAGGAGACC is a *cis*-acting element required for both anchoring and translational timing of *cyclin B1* mRNA.

Sufficiency of the CAGGAGACC element for mRNA anchoring

We previously reported that a reporter mRNA containing the *cyclin B1* 5' UTR, *gfp* ORF and *cyclin B1* 3' UTR but not *cyclin B1* ORF (named *tg3'*) failed to anchor at the animal polar cytoplasm (Yasuda et al. 2010). We examined the sufficiency of the CAGGAGACC element for mRNA anchoring by injection into oocyte nuclei of a variant form of *tg3'*, in which CAGGAGACC was inserted immediately before the stop codon of *gfp* (*tgcis-3'*, Fig. 2K). Although the percentage of oocytes having aggregated *tgcis-3'* signals seemed to be smaller than that of *tgo3'*, *524-1197* and *524-736*, it was evident that the *tgcis-3'* mRNA anchored at the animal polar cytoplasm (Fig. 2J; Table 1), indicating that the CAGGAGACC element can confer mRNA anchoring.

Linkage of actin filament-dependent aggregation to translational repression of mRNA

Identification of the 9-nt sequence as a dual *cis*-acting element necessary for both correct anchoring (aggregation) and accurate timing of translational activation of *cyclin B1* mRNA during oocyte maturation suggests a functional link between the two mechanisms. Our previous studies using an actin filament-destabilizing drug, cytochalasin B, showed that behavior of *cyclin B1* mRNA is dependent on a network of actin filaments (Kondo et al. 2001). Consistent with this finding, the aggregation of *cyclin B1* mRNA failed to disperse when MIH-treated oocytes were further treated with an actin filament-stabilizing drug, jasplakinolide (Fig. 6A), and these oocytes did not undergo *de novo* synthesis of Cyclin B1 proteins and activation of Cdc2 proteins, processes indispensable for promoting oocyte maturation in zebrafish oocytes (Fig. 6E). Like endogenous *cyclin B1* mRNAs, jasplakinolide inhibited the MIH-induced dispersion of aggregation (Fig. 6B) and translational activation (Fig. 6C) of *tgo3'* mRNAs. However, the drug had no effect on the translational activation of $tgo^{M}3'$ mRNAs, which did not aggregate in the animal polar cytoplasm (Fig. 2I, 3H; Table 1, 2); the $tgo^{M}3'$ mRNAs were translated after MIH stimulation at a timing similar to that in jasplakinolide-untreated oocytes (Fig. 6D). These results suggest that the repression of *cyclin B1* mRNAs is achieved by two successive processes, the 3' UTR-dependent but actin filament-independent process and the 9-nt sequence- and actin filament-dependent process. When stopped at the first process, *cyclin B1* mRNAs are localize to the animal hemisphere of oocytes and translated at an earlier timing after

MIH stimulation. The first process must be a prerequisite for the second process, in which *cyclin B1* mRNAs are aggregated in the animal polar cytoplasm and translated at the accurate timing after MIH treatment, the process probably making a great contribution to the prevention of precocious translational activation of *cyclin B1* mRNA to assure the normal progression of oocyte maturation.

Association of proteins with the sequence CAGGAGACC in vitro

As a first step to elucidate the molecular mechanisms of *cyclin B1* mRNA anchoring and translational regulation mediated by the sequence CAGGAGACC, we examined whether this element interacts with sequence-specific RNA-binding proteins by electrophoretic mobility shift assays. RNA probes consisting of 524-574 nt of the zebrafish *cyclin B1* coding region and those carrying mutations in CAGGAGACC were incubated with extracts from full-grown oocytes. The wild-type probe showed a retarded mobility (upward shift) when incubated with the extracts, while the mutant probe did not (Fig. 7). It is likely that the upward shift of the wild-type probe was caused by an interaction with proteins, because it was inhibited when the extracts were treated with proteinase K. We also examined the binding activity of 567-617 nts of frog *cyclin B1* and 599-647 nts of mouse *cyclin B1*, which are equivalent to 524-574 nts of zebrafish *cyclin B1*. The wild-type frog and mouse RNA probes, but not the mutant probes, interacted with zebrafish oocyte extracts (Fig. 7). These results suggest that the CAGGAGACC element regulates the translation via a *trans*-acting protein(s) by a mechanism conserved in vertebrates.

Secondary structure of cyclin B1 mRNAs around the sequence CAGGAGACC

It is known that secondary structures of target RNAs are important for recognition by RNA binding-proteins (Bullock and Ish-Horowicz 2001; Jambhekar et al. 2005). Since zebrafish, frog and mouse *cyclin B1* mRNAs interacted with zebrafish oocyte proteins (Fig. 7), we examined whether these mRNAs have similar secondary structures around the sequence CAGGAGACC. However, the secondary structures predicted by the CentroidFold program (Sato et al. 2009) have no similarity (Fig. 8), suggesting that the primary RNA sequence of CAGGAGACC, rather than its secondary structure, is critical for recognition by *trans*-acting proteins.

Subcellular localization of cyclin A1 and B2 mRNAs

Similar to zebrafish *cyclin B1* mRNA, *cyclin B2* mRNA harbors the CAGGAGACC sequence, whereas *cyclin A1* mRNA does not. To obtain further insights into the role of CAGGAGACC in mRNA anchoring, we examined the localization of these mRNAs in zebrafish full-grown oocytes. Consistent with the presence of CAGGAGACC, *cyclin B2* mRNA co-localized to the animal polar cytoplasm with *cyclin B1* mRNA, while *cyclin A1* mRNA did not show clear subcellular localization (Fig. 9).

Discussion

Although temporally regulated translation of localized mRNAs has been recognized as an important and universal mechanism for the control of gene expression, its

molecular details remain largely unresolved because of the limitation in technology that enables precise determination of the time and site of translational activation of localized mRNAs in the cell. Our genetic experimental system in combination with real-time imaging of translational activation provides an insight into the molecular mechanisms of temporally regulated translation of *cyclin B1* mRNAs that localize to the animal polar cytoplasm of oocytes. We propose that an evolutionally conserved sequence, CAGGAGACC, within the coding region of vertebrate *cyclin B1* mRNA is responsible for the mRNA to be anchored at the correct sites in the oocyte cytoplasm and to be translationally activated at the accurate timing during oocyte maturation, providing a direct molecular link between the two events.

Function of CAGGAGACC sequence in cyclin B1 mRNA localization

Although mRNA localization is controlled by *cis*-acting elements usually located in its 3' UTR (Martin and Ephrussi 2009), recent studies have demonstrated that the coding sequences of *ASH1*, *ATP2* and *ABP140* mRNAs are involved in mRNA localization in yeast (Gonzalez et al. 1999; Garcia et al. 2009; Kilchert and Spang 2011). For example, the N-terminal fragment of ABP140 protein that includes the actin-binding domain must be synthesized to transport mRNA to the distal pole of the mother cell via actin filaments in budding yeast (Kilchert and Spang 2011). Similarly, *Dia1* mRNA localizes to the perinuclear endoplasmic reticulum in chicken embryonic fibroblasts depending on the translation of nascent peptides encoded by the mRNA (Liao et al. 2010). In striking contrast to these cases, zebrafish *cyclin B1* reporter

mRNAs that contain a stop codon downstream of *gfp* and upstream of the *cyclin B1* initiation codon localize to the animal polar cytoplasm of oocytes, similar to endogenous *cyclin B1* mRNA (Figs. 2-4). The reporter mRNAs produce TC-tagged GFP but not Cyclin B1 protein. It is thus apparent that the localization of *cyclin B1* mRNA is independent of the nascent protein product encoded by the mRNA.

We demonstrated that the sequence CAGGAGACC within the *cyclin B1* coding region is required for mRNA anchoring in the presence of the 3' UTR (Figs. 2I, 3H, 4H). In addition to this requirement, we also suggested the sufficiency of CAGGAGACC by demonstrating that *tg3'* mRNA, which was dispersed throughout the animal hemisphere of oocytes (Yasuda et al. 2010), was anchored at the animal polar cytoplasm when the CAGGAGACC sequence was introduced to the end of the *gfp* ORF (*tgcis-3'*, Fig. 2J). However, it seemed that the extent of anchoring of *tgcis-3'* mRNA was not comparable to that of *tgo3'*, *524-1197* and *524-736* mRNAs (Table 1). The position of CAGGAGACC in the coding region may be important for the element to function properly.

A *trans*-acting protein(s) may mediate the anchoring, because RNA probes carrying the CAGGAGACC sequence, but not those carrying mutated sequences, interacted with oocyte proteins. As well as a zebrafish RNA probe, corresponding RNA probes derived from frog and mouse *cyclin B1* mRNAs also interacted with zebrafish oocyte proteins (Fig. 7), implying generality of the CAGGAGACC-mediated anchoring mechanism of *cyclin B1* mRNA in vertebrate oocytes. Taken together, our results suggest the following scenario leading to the localization of *cyclin B1* mRNA to the animal polar cytoplasm:

The 3' UTR dictates the mRNA to be transported to the cytoplasm beneath the animal pole, and then the 9-nt sequence in the coding region directs the transported mRNA to be anchored there with the aid of a *trans*-acting RNA-binding protein(s).

Since the sequence CAGGAGACC is distinct from any *cis*-acting elements responsible for mRNA behavior so far recognized (Lewis et al. 2004; see also review, Chabanon et al. 2004), it is difficult to predict proteins bound to this sequence specifically. Recent studies have demonstrated that secondary structures of target RNAs are important for recognition by RNA-binding proteins (Bullock and Ish-Horowicz 2001; Jambhekar et al. 2005). However, computationally predicted secondary structures of zebrafish, frog and mouse *cyclin B1* mRNAs have no apparent similarity (Fig. 8). We therefore speculate that the primary RNA sequence of CAGGAGACC, not its secondary structure, is important to function as a *cis*-acting element. To understand molecular details, biochemical identification of proteins bound to the sequence CAGGAGACC is in progress.

Function of CAGGAGACC sequence in temporally regulated translation

Temporally regulated translation of localized mRNA is crucial for various biological processes, including gametogenesis, embryonic development and neuronal plasticity (Mendez and Richter 2001; Martin 2004). Recent studies have revealed several *cis*-acting elements responsible for mRNA localization, including a 395-nt sequence in the 3'UTR of *vasopressin* mRNA for localization to neuronal dendrites (Mohr et al. 2001) and a 66-nt element in the 5' UTR of *sensorin* mRNA for localization to neuronal

synapses (Meer et al. 2012). These localized mRNAs are translationally activated in response to neuronal stimuli, but it is uncertain whether the localization is a prerequisite for translational activation.

Taking advantage of our genetic experimental system in combination with real-time imaging of translation, we comprehensively analyzed the relationship between *cyclin B1* mRNA localization and temporal control of translation after induction of oocyte maturation. In the presence of 5' and 3' UTRs, the 524-736 sequence in the *cyclin B1* coding region was sufficient to assure correct anchoring at the animal polar cytoplasm and accurate timing of translational activation after induction of oocyte maturation (Figs. 4E, 5D). We identified the core sequence involved in both the spatial and temporal regulation as CAGGAGACC, indicating that the localization of *cyclin B1* mRNA is tightly linked to the timing of translational activation during oocyte maturation via a single *cis*-acting element, CAGGAGACC. Experiments using the actin filament-stabilizing drug jasplakinolide also provided an insight into the relationship between mRNA localization and translational timing. When oocytes having an aggregation of *tgo3'* mRNAs were treated with jasplakinolide, neither MIH-induced dispersion of the aggregation nor translational activation of the mRNAs occurred, as in the case of endogenous *cyclin B1* mRNA. In striking contrast to this, *tgo^{M}3'* mRNAs, which do not form an aggregation in the animal polar cytoplasm, were translated at the original timing (earlier timing than that of endogenous and *tgo3'* mRNAs) even in the presence of jasplakinolide (Fig. 6). These findings highlight the significance of the CAGGAGACC- and actin filament-dependent localization (aggregation) of *cyclin B1*

mRNA in the translational timing during zebrafish oocyte maturation.

Function of evolutionally conserved coding sequence in mRNA regulation

In general, evolutionally conserved coding sequences encode evolutionally conserved amino acid sequences. Indeed, the sequence CAGGAGACC encodes evolutionally conserved amino acids Gln, Glu and Thr (Fig. 1C). However, it should be noted that the sequence CAGGAGACC is completely conserved in vertebrates despite the fact that each codon can be replaced with CAA (first codon), GAA (second codon) and ACA, ACG or ACU (third codon). The conservation of CAGGAGACC is not due to a preference of the codon usage for Gln, Glu and Thr. Human Cyclin B1 protein possesses 19 Glns, but the number of Glns encoded by CAG is only 9 (47.3%). Similarly, the numbers of Glus and Thrs in human Cyclin B1 protein are 33 and 23, respectively, but the number of Glus encoded by GAG and Thr by ACC are 10 (30.3%) and 3 (13%), respectively. Besides encoding conserved amino acid sequences, the sequence CAGGAGACC itself might have a role in the regulation of mRNA localization and translation in a wide range of vertebrate species.

We found that *cyclin B1* and *cyclin B2* mRNAs, both of which contain the 9-nt sequence, localized to the animal polar cytoplasm but that *cyclin A1* mRNA lacking the sequence did not (Fig. 9), confirming the correlation between presence of the 9-nt sequence and occurrence of mRNA anchoring. Besides *cyclin B1* and *B2* mRNAs, however, the animal polar cytoplasm of zebrafish full-grown oocytes harbors *cth1*, *mos*, *notch1a*, *pabp*, *pou-2*, *taram-a*, *vg1* and *zorba* mRNAs (Howley and Ho, 2000; Suzuki

et al. 2009), which do not have the 9-nt sequence. It is thus apparent that the 9-nt sequence is not essential for mRNA anchoring at the animal polar cytoplasm. A BLAST search for the existence of transcripts that have the sequence CAGGAGACC revealed that they account for 4.4%, 9.4%, 8.5% and 11.8% of zebrafish, frog, mouse and human transcripts. The transcripts encode various proteins with diverse functions, and their subcellular localizations remain to be elucidated. Further studies are necessary to evaluate the prevalence of the 9-nt sequence-mediated mechanism in vertebrates.

Conclusions

Translational activation of *cyclin B1* mRNAs plays a crucial role in oocyte maturation, but its molecular mechanism remains to be elucidated. We identified a novel 9-nt sequence, CAGGAGACC, in the coding region of *cyclin B1* mRNA as a *cis*-acting element involved in both correct localization of mRNA in the animal polar cytoplasm of zebrafish oocytes and in translational activation at the accurate timing during oocyte maturation. Since the 9-nt element is highly conserved in the coding region of vertebrate *cyclin B1* mRNA, the mechanism mediated by this element might function commonly in vertebrates.

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Figure legends

Fig. 1. Structures of reporter genes. (A) All reporter genes contain *cyclin B1* 5' UTR (5' UTR), coding sequences of TC-tag (TC) and EGFP (GFP), a stop codon and *cyclin B1* 3'UTR (3'UTR). The full length and various parts of the *cyclin B1* coding region were inserted downstream of the stop codon and upstream of the 3'UTR. The numbers of nucleotides (nts) of the *cyclin B1* coding sequence are indicated on the left. The *tgo*^{M}*3'* reporter contains mutations (indicated by red characters) in the CAGGAGACC sequence in the *cyclin B1* coding region (arrow). All of the reporter genes were transcribed under the control of the *Xenopus* EF1α promoter. The results of *in situ* hybridization (Aggregation) and real-time imaging of translation (Timing of translation) of reporter mRNAs transcribed from each reporter gene are summarized on the right. (B) Alignment of the coding sequences of zebrafish *cyclin B1* (GenBank accession: NM_131513) at 524-736 nts with the corresponding sequences of human (NM_031966), mouse (NM_172301) and frog (NM_001086727) *cyclin B1*. Asterisks indicate identical nucleotides. The blue box indicates the highly conserved 9-nt sequence. (C) Amino acid sequences encoded by the sequences are highlighted with grey and blue boxes in B.

Fig. 2. Localization of *cyclin B1* reporter mRNAs that were transcribed from reporter genes injected into oocyte nuclei. (A) Whole-mount *in situ* hybridization of full-grown oocytes probed with *cyclin B1*. Aggregation of c*yclin B1* mRNAs is found in the region indicated by dotted lines (200 μm). (B-J) Whole-mount *in situ* hybridization probed with *gfp*. Shown are oocytes injected with distilled water (DW) (B), reporter

genes containing the full length (*tgo3'*, C), 1-523 (D), 524-1197 (E), 524-736 (F), 737-949 (G) and 949-1197 (H) nts of the *cyclin B1* coding region, *tgo^{M}3'* reporter gene (I) and *tgcis-3'* reporter gene (J). The oocytes injected with distilled water showed no signals (B). The *tgo3'*, 524-1197, 524-736 and *tgcis-3'* mRNAs were aggregated in the animal polar cytoplasm (C, E, F, J). In contrast, the 1-523, 737-949, 949-1197 and *tgo^{M}3'* mRNAs were dispersed in the animal hemisphere of oocytes (D, G, H, I). Arrows indicate the signals of reporter mRNAs. Bars, 100 μm. (K) Structure of the *tgcis-3'* reporter gene, which contains *cyclin B1* 5' UTR (5'UTR), coding sequences of TC-tag (TC) and EGFP (GFP), CAGGAGACC element (E), a stop codon and *cyclin B1* 3' UTR (3'UTR).

Fig. 3. Localization of *cyclin B1* reporter mRNAs in oocytes of transgenic zebrafish. Whole-mount *in situ* hybridization of full-grown oocytes probed with *cyclin B1* (A) or *gfp* (B-I). Shown are oocytes expressing *tgo3'* (B), 1-523 (C), 524-1197 (D), 524-736 (E), 737-949 (F), 949-1197 (G), *tgo^{M}3'* (H) and SV40 (I) mRNAs. The *tgo3'*, 524-1197 and 524-736 mRNAs were aggregated in the animal polar cytoplasm (B, D, E). In contrast, the 1-523, 737-949, 949-1197 and *tgo^{M}3'* mRNAs were dispersed in the animal hemisphere (C, F, G, H) and the SV40 mRNAs were dispersed throughout the oocyte (I). Arrows indicate signals of *cyclin B1* (A) and reporter mRNAs (B-H). Bars, 100 μm. (J) Structure of the SV40 reporter gene, which contains *cyclin B1* 5' UTR (5'UTR), coding sequences of TC-tag (TC) and EGFP (GFP), a stop codon, *cyclin B1* coding region and SV40 3' UTR (SV40). (K) Quantification of reporter mRNAs

(normalized to *β-actin* mRNA) in full-grown transgenic oocytes by real-time PCR. No statistically significant difference was found in the contents of reporter mRNAs. Error bars indicate mean ± s.e.m. (n = 3).

Fig. 4. Localization of *cyclin B1* reporter mRNAs in the animal polar cytoplasm of transgenic zebrafish oocytes. (A) Section *in situ* hybridization of a full-grown oocyte probed with *cyclin B1*. The *cyclin B1* mRNAs were localized to the animal polar cytoplasm of the oocyte beneath the micro-pile as an aggregation (arrow). (B-H) Section *in situ* hybridization probed with *gfp*, showing full-grown oocytes of transgenic zebrafish expressing *tgo3'* (B), 1-523 (C), 524-1197 (D), 524-736 (E), 737-949 (F), 949-1197 (G) and $tgo^{M}3'$ (H) mRNAs. The *tgo3'*, 524-1197 and 524-736 mRNAs were aggregated beneath the micro-pile (arrows) (B, D, E), whereas the 1-523, 737-949, 949-1197 and $tgo^{M}3'$ mRNAs had no signal (C, F, G, H). Arrowheads indicate the micro-pile. Dotted lines encircle aggregated mRNAs. Bars, 100 μm.

Fig. 5. Real-time imaging of temporally regulated translation. (A-G) Real-time imaging of temporally regulated translation of *tgo3′* (A), 1-523 (B), 524-1197 (C), 524-736 (D), 737-949 (E), 949-1197 (F) and $tgo^{M}3'$ mRNAs (G). The times after MIH stimulation are shown as standardized time T_{GVBD}. Arrows indicate ReAsH signals detected at the first time after MIH stimulation. Dotted circles indicate the GV. Bars, 100 μm. (H) Translational timings of the reporter mRNAs after MIH stimulation. Error bars indicate mean ± s.e.m. (n = 3 for 1-523, 524-736 and $tgo^{M}3'$; n = 5 for 949-1197; n

= 6 for 524-1197; n = 7 for tgo3', 737-949). Transgenes indicated by B are translated earlier than those indicated by A, with statistically significant difference ($P < 0.05$, Student's *t*-test).

Fig. 6. Effects of actin filament stabilization on *cyclin B1* mRNA aggregation and translational regulation. (A, B) Section *in situ* hybridization of wild-type oocytes probed with *cyclin B1* (A) and *tgo3'* mRNA-expressing oocytes probed with *gfp* (B). Oocytes were treated with jasplakinolide (+) or DMSO (-) and stimulated with (+) or without (-) MIH. The oocytes were fixed 140 min after MIH stimulation. Arrowheads indicate the micro-pile. Arrows indicate the signals of *cyclin B1* mRNA (A) and *tgo3'* reporter mRNA (B). (C, D) Real-time imaging of oocytes expressing *tgo3'* (C) or *tgo^{M}3'* mRNAs (D) treated with jasplakinolide and MIH. The times after MIH treatment (min) are shown. Bars, 100 μm. Dotted circles show the GV. Arrow indicates the initial translation signal. Similar results were obtained from six oocytes expressing *tgo3'* mRNAs and three oocytes expressing *tgo^{M}3'* mRNAs. (E) Anti-Cyclin B1 and anti-Cdc2 immunoblots of oocytes treated with jasplakinolide (+) or DMSO (-) in the presence (+) or absence (-) of MIH. Cdc2 protein is a loading control of this experiment. An arrow indicates an active form of Cdc2 (Kondo et al. 2001).

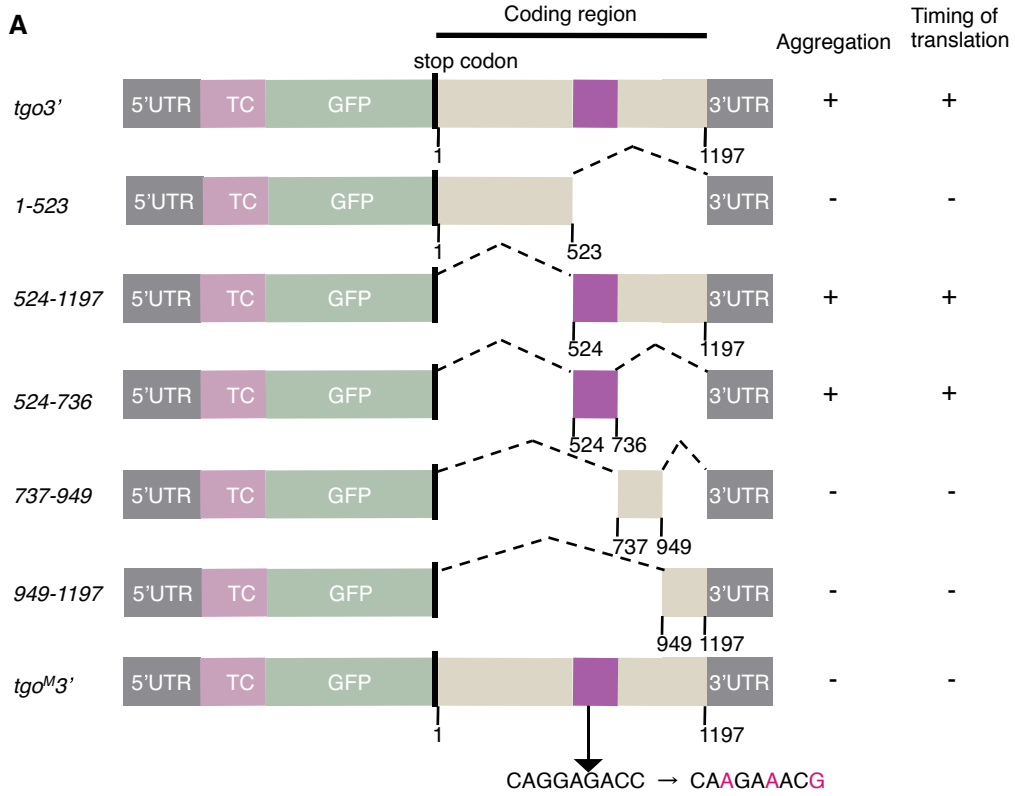
Fig. 7. Binding of proteins to CAGGAGACC. Electrophoretic mobility shift assay using RNA probes consisting of 524-574 nts of zebrafish *cyclin B1*, 567-617 nts of frog *cyclin B1* and 599-647 nts of mouse *cyclin B1* and corresponding mutant probes

(Zebrafish^{M}, Frog^{M}, Mouse^{M}). The RNA probes were incubated with binding buffer (Ext -) or extracts of 10 oocytes (Ext +). The reaction mixture was treated with (Pro K +) or without (Pro K -) proteinase K and loaded on 6% non-denaturing gels. The wild-type probes (but not the mutant probes) exhibit an upward shift (arrow) that was inhibited by proteinase K treatment. Asterisk indicates free RNA probes.

Fig. 8. Secondary structures of vertebrate *cyclin B1* mRNAs around the CAGGAGACC sequence. Secondary structures of zebrafish c*yclin B1* (524-736 nts, A), frog c*yclin B1* (521-733 nts, B) and mouse c*yclin B1* (599-811 nts, C) were computationally predicted. The possibility of nucleotide pairing is shown by color gradation (high in red and low in blue). The CAGGAGACC sequence is encircled by a magenta line.

Fig. 9. Localization of *cyclin* mRNAs in zebrafish oocytes. Serial sections (A and B, C and D) of a full-grown oocyte were subjected to *in situ* hybridization analysis with *cyclin B1* (A, C), *cyclin B2* (B) and *cyclin A1* (D) probes. Note the co-localization of *cyclin B1* and *B2* mRNAs, but not *cyclin A1* mRNA, to the animal polar cytoplasm. Bars, 100 μm.

Yasuda et al., Figure 1



B

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Human       623 GGCTAGTACAGGTTCAAATGAAATTCAGGTTGTTGCAGGAGACCATGTACATGAC  677
Mouse       599 GGCTAATACAGGTTCAGATGAAATTTAGGCTGCTTCAGGAGACCATGTACATGAC  653
Frog        567 GGCTGGTCCAGGTGCAAATGAAGTTCCGTCTGCTGCAGGAGACCATGTTTATGAC  621
Zebrafish   524 GGCTTGTGCAAGTCCAGATTAAGTTTAGGCTGCTTCAGGAGACCATGTACATGAC  578
                **** * ** ** ** ** ** **  *  ** * ***********  *****

Human       678 TGTCTCCATTATTGATCGGTTCATGCAGAATAATTGTGTGCCCAAGAAGATGCTG  732
Mouse       654 TGTGTCCATTATTGATCGGTTCATGCAGAACAGTTGTGTGCCCAAGAAGATGCTA  708
Frog        622 TGTTGGCATTATTGACCGTTTTCTGCAGGAACACCCAGTTCCCAAAAACCAGCTG  676
Zebrafish   579 TGTTGCCATCATCGATCGCTTCCTTCAGGATCATCCAGTTCCAAAGAAGCAGCTG  633
                ****  *** ** ** ** **  * *** *       ** ** ** **   ***

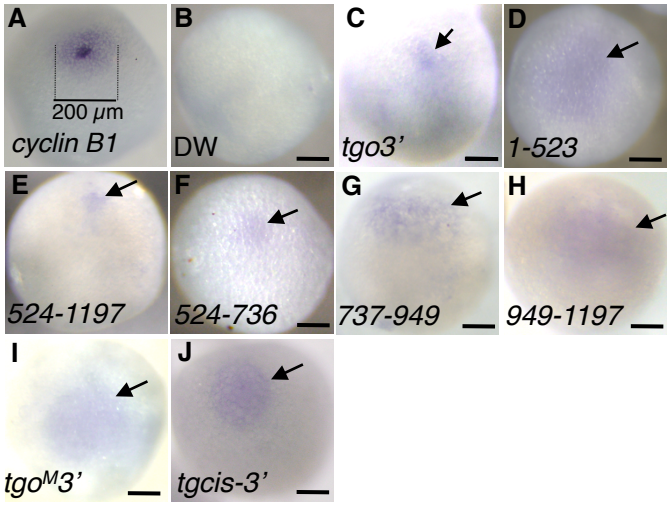
Human       733 CAGCTGGTTGGTGTCACTGCCATGTTTATTGCAAGCAAATATGAAGAAATGTACC  787
Mouse       709 CAGCTGGTCGGTGTAACGGCCATGTTTATTGCAAGCAAATATGAGGAGATGTACC  763
Frog        677 CAGCTTGTGGGGGTCACCGCAATGTTCCTTGCTGCTAAATACGAAGAGATGTACC  731
Zebrafish   634 CAACTTGTTGGTGTAACGGCCATGTTCATCGCCTCAAAATATGAAGAAATGTACC  688
                ** ** ** ** ** ** ** *****  *  *    ***** ** ** *******

Human       788 CTCCAGAAATTGGTGACTTTGCTTTTGTGACTGACAACACTTATACTA     835
Mouse       764 CTCCAGAAATAGGTGACTTCGCCTTTGTGACTAACAACACGTACACTA     811
Frog        732 CACCAGAAATTGGGGACTTTACTTTTGTAACTGATCACACATACACAA     770
Zebrafish   689 CACCAGAGATTGCAGACTTTGCTTTTGTGACAGACCGTGCATACACCA     736
                * ***** ** *  *****  * ***** **  *     * ** ** *
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C

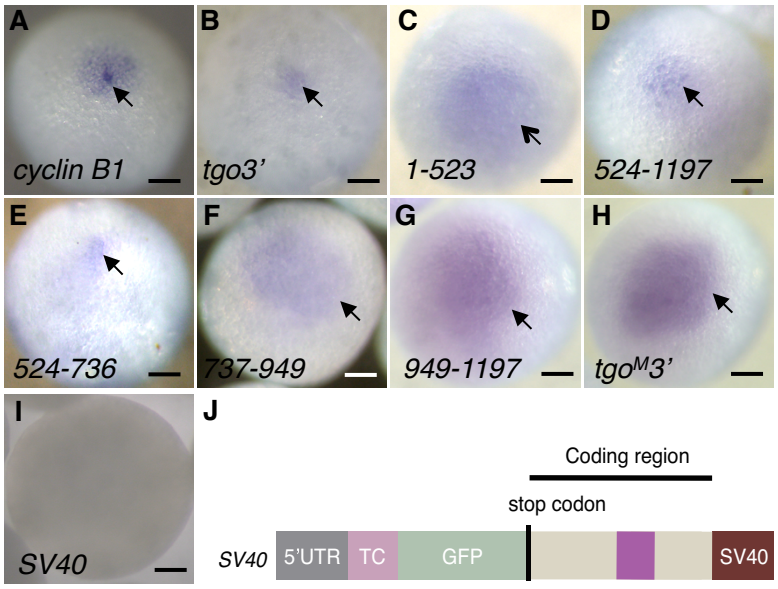
```
Human       RLLQETMYM
Mouse       RLLQETMYM
Frog        RLLQETMFM
Zebrafish   RLLQETMYM
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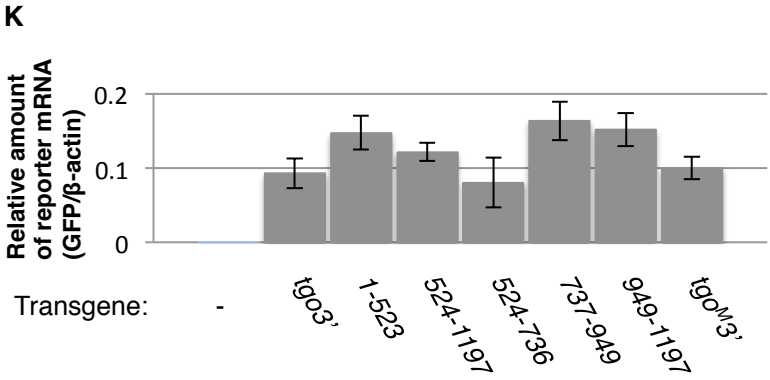
Yasuda et al., Figure 2



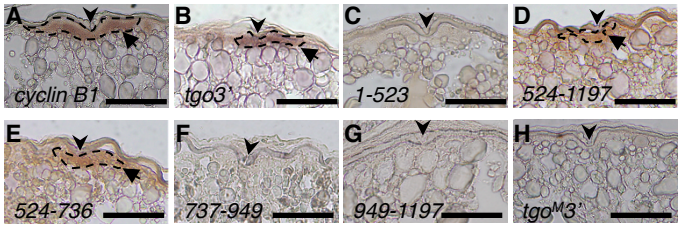


Yasuda et al., Figure 3

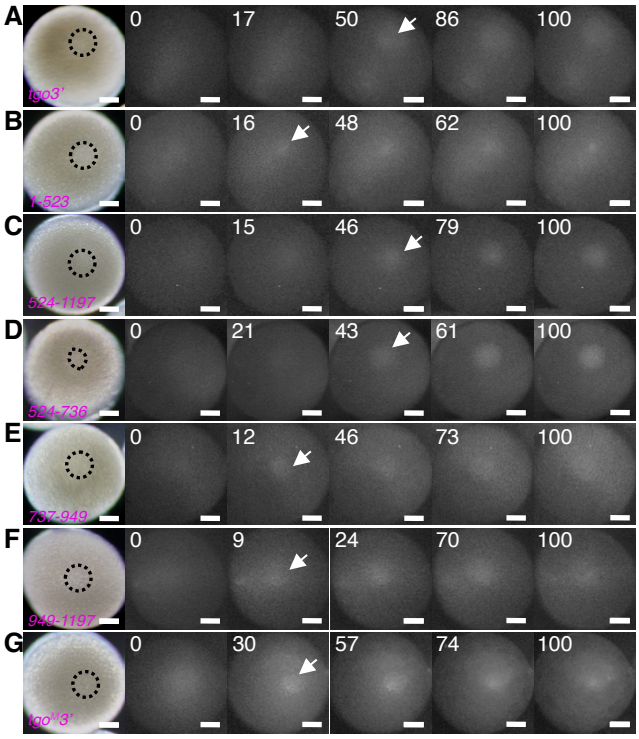


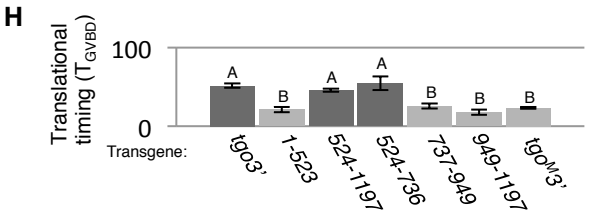


Yasuda et al., Figure 4

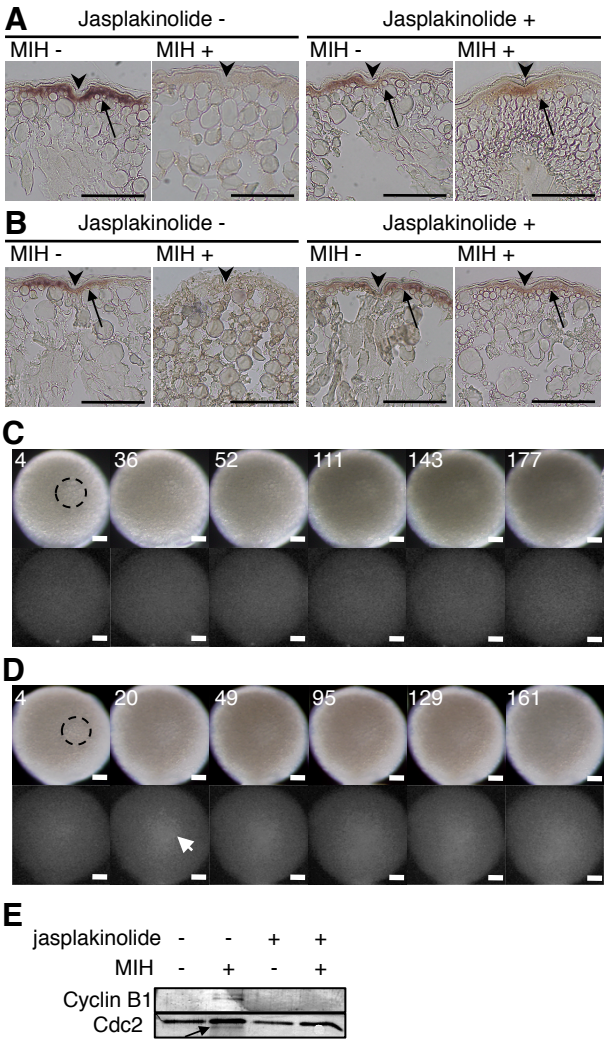


Yasuda et al., Figure 5

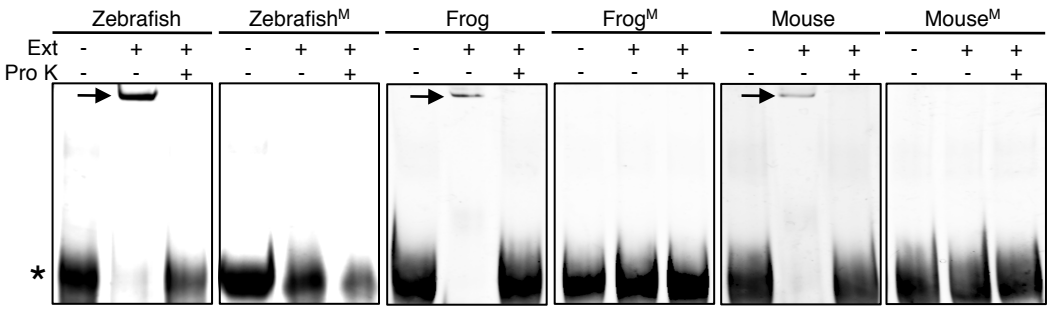




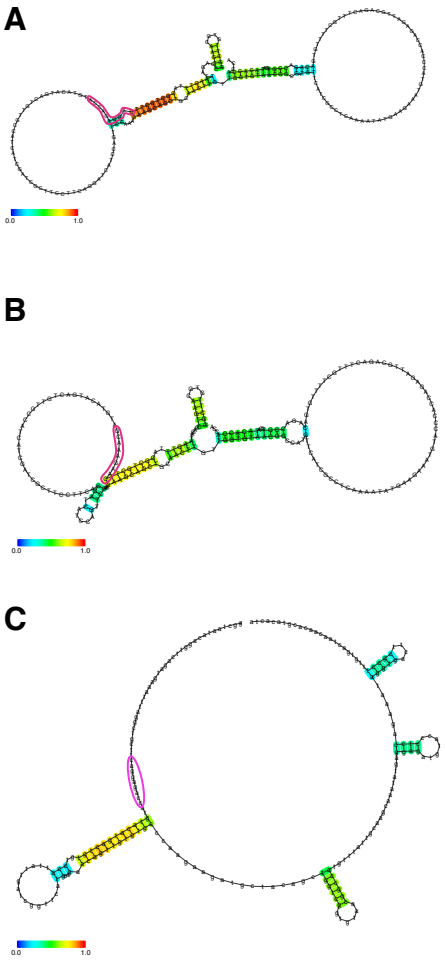
Yasuda et al., Figure 6



Yasuda et al., Figure 7



Yasuda et al., Figure 8



Yasuda et al., Figure 9

