<table>
<thead>
<tr>
<th>Title</th>
<th>Transgenic zebrafish reveals novel mechanisms of translational control of cyclin B1 mRNA in oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Yasuda, Kyota; Kotani, Tomoya; Ota, Ryoma; Yamashita, Masakane</td>
</tr>
<tr>
<td>Citation</td>
<td>Developmental Biology, 348(1), 76-86</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2010-12-01</td>
</tr>
<tr>
<td>Doc URL</td>
<td><a href="http://hdl.handle.net/2115/44425">http://hdl.handle.net/2115/44425</a></td>
</tr>
<tr>
<td>Type</td>
<td>article (author version)</td>
</tr>
<tr>
<td>File Information</td>
<td>DB348-1_76-86.pdf</td>
</tr>
</tbody>
</table>

Hokkaido University Collection of Scholarly and Academic Papers: HUSCAP
Transgenic zebrafish reveals novel mechanisms of translational control of cyclin B1 mRNA in oocytes

Kyota Yasuda\textsuperscript{a, c}, Tomoya Kotani\textsuperscript{b, c}, Ryoma Ota\textsuperscript{a}, and Masakane Yamashita\textsuperscript{b,*}

\textsuperscript{a} Laboratory of Reproductive and Developmental Biology, Biosystems Science Course, Graduate School of Life Science, Hokkaido University, Sapporo 060-0810, Japan
\textsuperscript{b} Laboratory of Reproductive and Developmental Biology, Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan
\textsuperscript{c} These authors contributed equally to this work.

*Corresponding author:
Department of Biological Sciences, Faculty of Science, Hokkaido University,
North 10 West 8, Sapporo, Hokkaido 060-0810, Japan
Tel.: +81-11-706-4454
Fax.: +81-11-706-4456
E-mail: myama@sci.hokudai.ac.jp
ABSTRACT
Temporal translation control of localized mRNA is crucial for regulating various cellular and developmental processes. However, little is known about mechanisms of temporal translation control of localized mRNA due to the limitation in technology. *cyclin B1* mRNA at the animal polar cytoplasm of immature zebrafish oocytes is translationally repressed, and its activation is temporally regulated during maturation. Mechanisms of *cyclin B1* translation in oocytes were analyzed using transgenic zebrafish in which reporter mRNAs are produced from transgenes introduced into the genome through transcription in the nucleus followed by transport to the cytoplasm, as in endogenous mRNAs. Real-time imaging of the site and timing of translation showed that mRNAs containing the full-length *cyclin B1* sequence precisely mimic the localization and translation of endogenous *cyclin B1* mRNA. However, mRNAs containing *cyclin B1* 3' untranslated region but lacking open reading frame (ORF) underwent abnormal localization and precocious translational activation, indicating the significance of the ORF in translational control of *cyclin B1* mRNA. Our genetic approach in combination with real-time imaging of the translation site and timing provides a novel insight into mechanisms of temporal control of translation.

Keywords: Translational control, Oocyte maturation, Cyclin B1, Transgenic zebrafish, Real-time imaging
Introduction

mRNAs localized to particular regions in the cell are translated at precise timings at which their protein products are needed. This spatio-temporally regulated gene expression in the cell is critical for various cellular and developmental processes, including the targeting of protein synthesis to particular subcellular domains, formation of body patterns and specification of cell fate (Kloc et al., 2002; St Johnston, 2005). Control of gene expression by mRNA localization had been thought to be a mechanism that is specific to a small number of genes in limited cell types. However, recent comprehensive studies on mRNA localization have demonstrated that 71% of 2314 mRNAs expressed in *Drosophila* embryos exhibit definite subcellular localization within the cells (Lecuyer et al., 2007) and that ~5% and ~10% of all mRNAs present in *Xenopus* eggs and mitotic human cells, respectively, are associated with mitotic microtubules (Blower et al., 2007). These findings imply that mRNA localization is a conserved and widely used mechanism for controlling gene expression in a variety of cell types.

In general, the localization and translational control of mRNAs are linked to each other, and they are controlled by *cis*-acting elements in the 3’ untranslated region (UTR) of mRNAs. The *cis*-acting elements are recognized by *trans*-acting factors that direct intracellular localization and mediate translational repression and activation of target mRNAs (Martin and Ephrussi, 2009). In theory, translational control of localized mRNAs can be divided into two modes: 1) spatial control, in which translation is repressed until the mRNA reaches its final destination and activated at the site where the mRNA is localized and 2) temporal control, in which translation remains repressed even after the mRNA has reached its final destination and is activated at the time when protein products are required, irrespective of the timing of localization (Besse and Ephrussi, 2008). Spatially controlled translation of localized mRNAs has been well-documented for *ASH1* mRNA in yeast cells (Chartrand et al., 2002; Long et al., 1997; Paquin et al., 2007; Takizawa et al., 1997), *nanos* mRNA in *Drosophila* embryos (Dahanukar et al., 1999; Gavis and Lehmann, 1994; Smibert et al., 1996; Zaessinger et al., 2006) and *β*-actin mRNA in mammalian cells (Huttemaier et al., 2005). In contrast, molecular mechanisms underlying temporally controlled translation of localized mRNAs remain poorly understood because of the limitation in technology that enables precise determination of the time and site of translational activation in the cell.
Oocytes stop their meiotic cell cycle at prophase I (prophase I arrest), during which time they grow due to the accumulation of substances necessary for early embryonic development (vitellogenesis). In many vertebrates, full-grown postvitellogenic oocytes under prophase I arrest are unable to be fertilized until they mature in response to maturation-inducing hormone (MIH) (Nagahama and Yamashita, 2008). Normal progression of oocyte maturation, including the meiotic cell cycle from prophase I to metaphase II, requires temporally controlled translation of mRNAs that are stored as translationally repressed forms in prophase I-arrested immature oocytes. For example, Mos, Cyclin B and Wee1 must be synthesized in that order, and the protein syntheses at correct timings are important for the initiation of oocyte maturation and the transition between meiosis I to II without intervening DNA replication (Furuno et al., 1994; Haccard and Jessus, 2006; Kotani and Yamashita, 2002; Ledan et al., 2001; Nakajo et al., 2000; Sagata et al., 1989). Mechanisms of translational control of these mRNAs have been extensively analyzed using *Xenopus* since it is fecund and oocytes are easy to handle *in vitro*. These characteristics have enabled straightforward experiments in which reporter mRNAs with various cis-acting elements were *in vitro*-transcribed from cDNAs and injected directly into the oocyte cytoplasm or mixed in oocyte extracts to react and then polyadenylation states, protein products or trans-acting factors were analyzed biochemically (for review, Radford et al., 2008). Although it has been proven that *Xenopus* oocytes provide an extremely powerful tool for understanding the molecular mechanisms of translational control, recent studies have demonstrated that the association of mRNAs with nuclear factors subsequently to their transcription in the nucleus is a prerequisite for accurate translational regulation in the cytoplasm (Kress et al., 2004; Lin et al., 2010; Shen et al., 2009; see also review, Kloc and Etkin, 2005). In addition to biochemical analyses with *in vitro*-transcribed exogenous mRNAs, translational control of mRNAs should be analyzed by a genetic approach that enables the production of reporter mRNAs in oocytes through transcription in the nucleus as in native mRNAs.

In fish and amphibians except *Xenopus*, Cyclin B1 proteins are absent in immature oocytes and de novo synthesis of Cyclin B1 by translational activation of stored mRNAs is essential for initiation of oocyte maturation (Ihara et al., 1998; Kondo et al., 1997; Tanaka and Yamashita, 1995; see also reviews, Nagahama and Yamashita, 2008; Yamashita et al., 2000). We previously found that *cyclin B1* mRNA is localized in the
cortical cytoplasm beneath the animal pole of zebrafish oocytes as an actin filament-dependent aggregation (Kondo et al., 2001). The aggregation of cyclin B1 mRNA is dispersed prior to germinal vesicle breakdown (GVBD) during oocyte maturation, in association with the synthesis of Cyclin B1 protein. Treatment of immature oocytes with an actin filament-depolymerizing agent, cytochalasin B, results in disruption of the aggregation into small parts throughout the cortical cytoplasm and in prevention of MIH-induced oocyte maturation, implying that the temporally controlled local synthesis of Cyclin B1 protein is important for progression of oocyte maturation (Kondo et al., 2001).

To understand the molecular mechanisms underlying temporally controlled translation of localized cyclin B1 mRNAs during zebrafish oocyte maturation, we designed a genetic analysis in which reporter genes were introduced into the zebrafish genome using Tol2 transposon-mediated germ-line transmission and transcribed in oocytes (Kawakami et al., 2004; Kotani and Kawakami, 2008; Kotani et al., 2006). Moreover, a real-time imaging method that was developed to visualize spatially controlled translation of β-actin mRNA in cultured cells (Rodríguez et al., 2006) was applied to our study. This method relies on the reaction that a biarsenial dye, ReAsH, emits fluorescence immediately after its binding to a nascent tetracysteine (TC)-tagged protein during translation, and therefore precise detection of the time and site of translational activation of reporter mRNAs is possible.

Here, we show that a reporter mRNA containing the open reading frame (ORF) and 3'UTR of cyclin B1 is localized to the animal pole of oocytes to form an aggregation, similar to endogenous cyclin B1 mRNA. Real-time imaging reveals that this reporter mRNA is translationally activated at timing similar to that of endogenous cyclin B1 mRNA. In contrast, a reporter mRNA lacking the ORF of cyclin B1 accumulates at the animal pole but does not aggregate there. Furthermore, this reporter mRNA is translated precociously after MIH stimulation. Thus, our results demonstrate the requirement of ORF sequences not only for mRNA localization but also for temporal control of mRNA translation. We also found that reporter mRNAs harboring the same cis-elements behave differently in oocytes depending on the process of their preparation: the reporter mRNA containing the ORF and 3'UTR of cyclin B1 in the transgenic zebrafish, which was transcribed in the nucleus and transported to the cytoplasm, exhibits behavior similar to that of endogenous cyclin B1 mRNA; however, despite the presence of the same
cis-elements, the reporter mRNA does not mimic the localization and temporal translation of endogenous cyclin B1 mRNA when it was transcribed in vitro and injected into the oocyte cytoplasm. This finding indicates that exogenous mRNAs without any experience in the nucleus are not suitable for elucidating the mechanisms of temporal control of translation, at least in zebrafish oocytes. We believe that our genetic approach in combination with real-time imaging of the site and timing of translation of reporter mRNAs discloses mechanisms that have been overlooked by previous biochemical analyses, providing new dimensions in the study of molecular mechanisms by which translation of mRNAs is spatially and temporally regulated.

Materials and methods

Construction of reporter genes

The 5’UTR, ORF and 3’UTR sequences of zebrafish cyclin B1 mRNA and the sequence encoding a TC-tag were amplified by PCR with a full-length zebrafish cyclin B1 cDNA (Kondo et al., 1997) and the following primers. The 5’UTR of cyclin B1 and the sequence encoding a TC-tag were amplified with cycB1-5’UTR-f1 (5’-CAC CGG ATC CGG CAC GAG TAA ACC TCT CTG TA-3’) (underline indicating BamHI site) and cycB1-5’UTR-r1-TC (5’-TTA GAT CTG TTG GCT CCA TAC AAC ATC CTG GGC AGC AGT TGA GGA ACA TGG TTC CGG ATT CTC GTG GTT TAT TGC TGT G-3’) (underline indicating BglII site and double-underline indicating the sequence encoding TC-tag). The ORF of cyclin B1 was amplified with cycB1-ORF-f1 (5’-TTC GAA ATG ATG GCT CTC CGG TGT C-3’) and cycB1-ORF-r1 (5’-TTC GAA CTA TGA GGA TCT GCT TAG CC-3’) (underlines indicating BstBI site). The 3’UTR of cyclin B1 was amplified with cycB1-3’UTR-f1 (5’-GGA TCG ATT TGG GGT TAT GCT GAA G-3’) (underline indicating ClaI site) and cycB1-3’UTR-r1 (5’-GAA GAT CTA AAA CTT TAA AAA GTT ATT G-3’) (underline indicating BglII site). The PCR products of 5’UTR, ORF and 3’UTR of cyclin B1 were inserted into BamHI, ClaI, and ClaI/BglII sites of T2KXIGΔin (Urasaki et al., 2006), respectively.

Production of transgenic zebrafish

All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University. Transgenic zebrafish was produced using Tol2 transposon-mediated germ-line transmission (Kotani et al., 2006). One nl of a solution
containing 250 µg/ml of the reporter constructs and 250 µg/ml of in vitro-synthesized mRNA 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 a fluorescence stereomicroscope MZFLIII (Leica). The embryos expressing GFP were raised to adulthood and the ovaries from F1, F2 and F3 females were used for in situ hybridization analysis, real-time imaging and polyadenylation assay.

Preparation of oocytes

Oocytes were manually isolated from ovaries with two pairs of forceps in zebrafish Ringer’s solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, 5 mM HEPES; pH 7.2) under a dissecting microscope. The oocytes were classified into five stages according to their diameters (stage I, 180-300 µm; stage II, 300-430 µm; stage III, 430-550 µm; stage IV, 550-670 mm) (Selman et al., 1993).

Whole-mount and section in situ hybridization analyses

For whole-mount in situ hybridization analysis, stage IV oocytes were manually isolated and fixed with 4% paraformaldehyde in phosphate buffered saline (4% PFA/PBS) (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4; pH 7.2) overnight at 4˚C. The hybridization and detection of the signal were performed according to the procedure reported previously (Schulte-Merker et al., 1992). Section in situ hybridization analysis 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) according to the manufacturer’s instructions. Total RNAs extracted from stage IV oocytes derived from wild-type and transgenic fish were used for cDNA synthesis using Super Script III First Strand Synthesis System (Invitrogen). GFP and β-actin transcripts were amplified with the cDNAs and the following primer sets: GFP-f1 (5’-CGA CCA CTA CCA GCA GAA CA-3’) and GFP-r1 (5’-ACG AAC TCC AGC AGG ACC AT-3’) and β-actin-f1 (5’-AAA TCG CTG CCC TGG TCG TT-3’) and β-actin-r1 (5’-CTG
TCC CAT GCC AAC CAT CA-3’). The content of \textit{gfp} reporter mRNAs was normalized to that of \(\beta\)-actin mRNA. No \textit{gfp} reporter mRNAs were detected when oocyte cDNAs from wild-type fish were used.

\textit{Real-time imaging}

To verify that translation of reporter mRNAs encoding TC-tagged GFP can be visualized by ReAsH, we first observed cleavage-stage embryos. After removal of the chorions manually, the embryos from wild-type and transgenic fish were incubated with 1 \(\mu\)M ReAsH dye (Invitrogen) in zebrafish Ringer’s solution for 70 min and observed under a Leica MZFLIII fluorescence stereomicroscope using a filter set of Texas Red to detect the fluorescent signals from ReAsH.

To detect translation of reporter mRNAs in oocytes, 1 nl of 0.2 mM ReAsH in zebrafish Ringer’s solution was injected into stage IV oocytes using a microinjector, CellTram vario (Eppendorf). Oocyte maturation was induced by treatment with 17\(\alpha\),20\(\beta\)-dihydroxy-4-pregnen-3-one, an MIH in fish. The ReAsH signal was photographed by using a cooled CCD color camera VB-7010 (Keyence) at intervals of 15 min after MIH stimulation. To confirm that ReAsH signals originate from translation of mRNAs, the oocytes were incubated with 40 \(\mu\)g/ml puromycin in zebrafish Ringer’s solution for 20 min to disrupt the polysomes involved in the translation.

\textit{Polyadenylation assay}

RNA ligation-coupled RT-PCR was performed according to the procedure reported previously (Charlesworth et al., 2004). Two \(\mu\)g of total RNA extracted from pools of 15 oocytes was ligated to 0.4 \(\mu\)g of P1 anchor primer (5’-P-GGT CAC CTT GAT CTG AAG C-NH2-3’) in a 10-\(\mu\)l reaction using T4 RNA ligase (New England Biolabs) at 37°C for 30 min. The ligase was inactivated at 92°C for 5 min. Half of the RNA ligation reaction was used in a 25-\(\mu\)l reverse transcription reaction using Superscript III First Strand Synthesis System with a P1’ primer (5’-GCT TCA GAT CAA GGT GAC CTT TTT-3’). Five \(\mu\)l of the cDNA was used for the 1st PCR with the P1’ primer and a \textit{cycB1}-ORF-f2 primer (5’-GAA GAA TAA GTA CTC CAG TCA G-3’) (for amplifying the \textit{cyclin B1} transcript) or GFP-ORF-f1 primer (5’-TAC CCC GAC CAC ATG AAG CA-3’) (for amplifying the transcripts of reporter genes) for 15 cycles. Two \(\mu\)l of the 1st PCR reaction was used for the 2nd PCR with the P1’ primer and a \textit{cycB1}-3’UTR-f2
primer (5’-TAC GGA TTT CTT CAC TGC CAT G-3’) for 25 cycles. The PCR product was resolved on a 2% TAE gel. The cycB1-3’UTR-f2 primer was designed to be 107 nucleotides from the poly(A) addition site. We confirmed that the increase in PCR product length is due to extension of the poly(A) tail by cloning the 2nd PCR products and sequencing them. No PCR product was obtained with oocyte cDNAs from tgs zebrafish, which expresses mRNAs without the cyclin B1 3’UTR, indicating that the PCR product is derived from tgo3’ and tg3’ mRNAs.

**Injections of in vitro-synthesized mRNA and dextran**

The tgo3’ gene was amplified by PCR with cycB1-5’UTR-f1 primer and cycB1-3’UTR-r2 primer (5’-CTC GAG AAA ACT TTA AAA AGT TTA TTT GAA-3’) (underline indicating XhoI site) and cloned into pGEM-T vector (Promega). The resulting plasmid pGEM-tgo3’ was linearized with XhoI, and tgo3’ mRNA was synthesized by using an mMESSAGE mMACHINE T7 Kit (Ambion). Wild-type full-grown oocytes were injected with 2 nl of tgo3’ mRNA (10 µg/ml), which yielded a concentration equivalent to that of the tgo3’ transcript in transgenic oocytes. The injected oocytes were fixed with 4% PFA/PBS at 1, 2 and 4 h after injection and used for whole-mount in situ hybridization analysis. As a control, 2 nl of fluorescein dextran (1 mg/ml, 5 x10^5 Mr, Molecular probe), comparable with the relative molecular mass of tgo3’ mRNA (7.2 x10^5 Mr), was injected into full-grown oocytes. The injected oocytes were observed under a fluorescence stereomicroscope and photographed at 1, 2 and 4 h after injection.

**Results**

**Requirement of cyclin B1 ORF sequence for mRNA aggregation**

To analyze the molecular mechanisms underlying localization and translational control of cyclin B1 mRNA, we constructed the following three reporter genes (Fig. 1): 1) cycB1 5’UTR-TC-GFP-cycB1 ORF 3’UTR, hereafter referred to as tgo3’ gene, which contains cyclin B1 5’UTR, protein coding sequences of TC-tag and EGFP followed by a stop codon, cyclin B1 ORF and cyclin B1 3’UTR (Since reporter mRNA transcribed from the tgo3’ gene contains the stop codon upstream of cyclin B1 ORF, it produces TC-tagged GFP protein but not Cyclin B1 protein, as do mRNAs from other transgenes described below); 2) cycB1 5’UTR-TC-GFP-cycB1 3’UTR, referred to as
tg3’, in which the cyclin B1 ORF was deleted from tgo3’; and 3) cycB1 5’UTR-TC-GFP-SV40 3’UTR, referred to as tgs, in which the cyclin B1 3’UTR of tg3’ gene was replaced with SV40 3’UTR. This reporter was used as a negative control, because SV40 3’UTR does not contain any localization sequences (Kislauskis et al., 1993). All constructs are expressed under the control of Xenopus EF1α promoter, which drives transcription of exogenous genes in zebrafish oocytes (Kotani and Kawakami, 2008). The reporter genes were then introduced into the zebrafish genome by Tol2 transposon-mediated germ-line transmission. Three lines carrying the same reporter gene were produced and at least 2 lines in each construct were analyzed. All lines carrying the same reporter gene gave equivalent results.

Consistent with our previous report on oocytes from wild-type zebrafish (Kondo et al., 2001), whole-mount in situ hybridization showed that endogenous cyclin B1 mRNA aggregated in the cortical cytoplasm beneath the animal pole of full-grown oocytes from transgenic zebrafish (Fig. 2A). We then analyzed the localization of reporter mRNAs by whole-mount in situ hybridization with a gfp probe. The tgo3’ mRNA harboring the ORF and 3’UTR of cyclin B1 aggregated in the cortical cytoplasm of full-grown oocytes as did endogenous cyclin B1 mRNA (Fig. 2B and Table 1), confirming that this reporter can precisely mimic the behavior of endogenous cyclin B1 mRNA. In contrast, the tgs mRNA, which has SV40 3’UTR but not cyclin B1 ORF and 3’UTR, was distributed throughout the cortical cytoplasm of oocytes (Fig. 2C and Table 1), indicating that the cyclin B1 sequence is necessary for mRNA aggregation. The tg3’ mRNA harboring the 3’UTR of cyclin B1 but lacking the ORF exhibited particularly interesting behavior: It did not form any aggregates but was widely distributed throughout the hemisphere of oocytes (Fig. 2D-F). We classified the localization patterns of tg3’ mRNA into three categories by the area occupied by signals: class I, signal restricted to less than 1/4 of the oocyte hemisphere (Fig. 2D); class II, signal ranging from 1/4 to 1/2 of the hemisphere (Fig. 2E); and class III, signal expanding over 1/2 of the hemisphere (Fig. 2F) (Table 1). Real-time PCR analysis showed that each transcript was accumulated at similar levels in full-grown oocytes (Fig. 2G), indicating that the difference in localization patterns is not due to the difference in expression levels of reporter mRNAs. Taken together, these results indicate that cyclin B1 ORF sequences are required for the mRNA aggregation in oocytes.
Cis-acting elements responsible for the aggregation of cyclin B1 mRNA

To analyze the localizing processes of reporter mRNAs during oogenesis, we performed section in situ hybridization of ovaries derived from transgenic zebrafish. As we reported previously (Kondo et al., 2001), cyclin B1 mRNA was localized to the animal pole of oocytes from stage III to full-grown stage IV (Fig. 3A, E). The tgo3' mRNA was found to be localized to the animal pole from stage III to stage IV (Fig. 3B, F), further supporting the notion that this reporter behaves similarly to endogenous cyclin B1 mRNA. The tg3' mRNA was also localized to the animal pole in stage III oocytes (Fig. 3C); however, the signals did not aggregate but decreased to undetectable levels in stage IV oocytes (Fig. 3G), probably due to dispersion of signals within the oocytes. The tgs mRNA showed no localization in any of the stages of oocytes (Fig. 3D, H). These results suggest that cyclin B1 mRNA is localized to the animal pole depending on its 3’UTR and stably anchored there depending on the ORF, resulting in the aggregation in full-grown oocytes.

Translational repression and activation of cyclin B1 mRNA through 3’UTR sequence

By observing GFP expression in oocytes, we examined whether reporter mRNAs are translationally regulated. No GFP signals were detected in immature and mature oocytes from wild-type zebrafish (Fig. 4A), while high levels of GFP expression were detected in immature and mature oocytes expressing tgs mRNAs (Fig. 4B). In oocytes expressing the tgo3’ or tg3’ mRNAs, GFP expression was not detected in immature oocytes but was detected in mature oocytes (Fig. 4C, D), indicating that these transcripts are translationally repressed in immature oocytes and activated during oocyte maturation. These results show that the cyclin B1 3’UTR is necessary and sufficient for translational repression in immature oocytes and translational activation during oocyte maturation.

Real-time imaging of temporally controlled cyclin B1 translation in oocytes

GFP proteins become visible under a fluorescence microscope only after being correctly folded, a process that requires a substantial period of time after translation from the mRNA (Cubitt et al., 1995). This means that GFP proteins have been diffused in the cell before their detection by fluorescence, therefore making it impossible to accurately determine the site and timing of translation of gfp reporter mRNAs in the cell.
by observing GFP expression. We overcame this problem by applying a real-time imaging method (Rodriguez et al., 2006) to our genetic analysis (Fig. 5A). The biarsenial dye ReAsH specifically binds to a TC-tag and immediately emits fluorescence after binding, allowing detection of the translation site. Although it has been reported that ReAsH visualizes spatially controlled translation of localized mRNAs in mammalian cultured cells (Rodriguez et al., 2006), it remains to be confirmed that this technique can capture temporally controlled translation in fish oocytes.

First, we examined whether ReAsH binds to TC-tagged GFP proteins and emits fluorescence in zebrafish cells using cleavage-stage embryos. The embryos were incubated in a staining solution containing ReAsH after being liberated from the impermeable chorions around them. ReAsH fluorescent signals were found in the embryos expressing TC-tagged GFP proteins (Fig. 5C) but not in the wild-type embryos (Fig. 5B), confirming that ReAsH can detect TC-tagged proteins in zebrafish cells. We then attempted to detect TC-tagged proteins in transgenic zebrafish oocytes by treating them with ReAsH; however, we failed to obtain a specific signal because of high levels of background signals in follicle cells surrounding the oocytes. To decrease the background signals, ReAsH was directly injected into oocytes to be analyzed. Maturation of the injected oocytes was induced by treatment with MIH and the oocytes were monitored by fluorescence microscopy during maturation. Since the time at which GVBD occurs in response to MIH varies from oocyte to oocyte (90–150 min after MIH stimulation), the time when the oocyte underwent GVBD (judged by disappearance of the nuclear envelope) was expressed as $T_{GVBD} = 100$ and the time of translation (judged by the appearance of ReAsH signal) was expressed relative to this time. ReAsH signals were not detected in wild-type oocytes throughout the course of maturation (data not shown), whereas the signals were detected at the animal pole of oocytes expressing $tgo3'$ mRNAs (Fig. 5D arrow) at the time $T_{GVBD} = 51.4 \pm 4.0 \ (n = 7, \ Fig. \ 5G)$. Treatment of oocytes with puromycin, which dissociates the polysomes responsible for the translation, decreased the ReAsH signals (Fig. 5F), confirming that the signals result from the translation of reporter mRNAs. To our knowledge, this is the first demonstration of real-time imaging of temporally controlled translation in living cells.
Requirement of ORF sequence for temporal control of cyclin B1 translation

We next analyzed the translation patterns of \( tg3' \) mRNAs. Translation of \( tg3' \) mRNAs was detected at the animal pole of oocytes at a timing (\( T_{GVBD} = 19.0 \pm 8.4, n = 4, p < 0.05, \) Fig. 5E, G) earlier than that of \( tgo3' \) mRNAs (approximately \( T_{GVBD} = 50, \) Fig. 5G). In addition, there were significant differences in the site of translation between \( tg3' \) and \( tgo3' \) mRNAs. In striking contrast to the translation site of \( tgo3' \) mRNAs, which remained in the animal polar cytoplasm, that of \( tg3' \) mRNAs expanded in accordance with the fact that the region occupied by the \( tg3' \) mRNA is wider than that occupied by the \( tgo3' \) mRNA (see Fig. 2D-F). These results reveal that the ORF sequence of cyclin B1 mRNA is required for the precise timing of translational activation of the mRNA.

Polyadenylation of cyclin B1 and reporter mRNAs during oocyte maturation

Translational activation of cyclin B1 mRNA is regulated by cytoplasmic polyadenylation during Xenopus oocyte maturation (Mendez and Richter, 2001). We examined the polyadenylation states of cyclin B1 mRNA during zebrafish oocyte maturation by RNA ligation-coupled RT-PCR (Charlesworth et al., 2004). As expected, a short poly(A) tail of about 50 nucleotides (nt) was detected in immature oocytes and an elongated poly(A) tail of about 50-200 nt was detected in mature oocytes (Fig. 6A), consistent with results reported previously (Zhang and Sheets, 2009). Zebrafish cyclin B1 mRNA is less efficiently polyadenylated than is Xenopus cyclin B1 mRNA because of differences in their 3'UTR sequences (Zhang and Sheets, 2009).

We then examined the polyadenylation states of \( tgo3' \) and \( tg3' \) mRNAs. Their poly(A) tails remained short in immature oocytes and were elongated in mature oocytes (Fig. 6A), suggesting that the translational activation of reporter mRNAs is regulated by cytoplasmic polyadenylation similar to that of endogenous cyclin B1 mRNA. We also examined the changes in poly(A) length in the process of oocyte maturation. Since we cannot follow the changes in poly(A) length in a single oocyte during maturation, 15 oocytes were collected at intervals of 15 min during maturation and the poly(A) lengths at each time point were examined. Since the time of GVBD varied in oocytes, the time when GVBD occurred in half of the oocytes was expressed as \( T_{GVBD_{50}} = 100, \) and the time when the polyadenylation occurred was expressed relatively. Polyadenylation of cyclin B1 mRNA was first detected at the time \( T_{GVBD_{50}} = 58.9 \pm 8.4 (n = 3) \) after MIH.
stimulation (Fig. 6B, C). Polyadenylation of tgo3’ mRNA occurred at the time $T_{\text{GVBD50}} = 58.9 \pm 8.4$ (n = 3), coincident with that of cyclin B1 mRNA (Fig. 6B, C), suggesting that the tgo3’ mRNA is translationally activated at a time similar to that of cyclin B1 mRNA. In contrast, polyadenylation of tg3’ mRNA was detected at the time $T_{\text{GVBD50}} = 18.1 \pm 6.4$ (n = 3, p < 0.05), which is earlier than those of cyclin B1 and tgo3’ mRNAs (Fig. 6B, C). Therefore, the ORF sequences are also required for temporal control of polyadenylation of cyclin B1 mRNA during oocyte maturation.

Requirement of nuclear events for mRNA localization and translational control

To confirm that nuclear events are necessary for localization and translational control of cyclin B1 mRNA, we injected in vitro-synthesized tgo3’ mRNA into the zebrafish oocyte cytoplasm and examined its localization and translational regulation. Whole-mount in situ hybridization analysis with a gfp probe showed that the in vitro-synthesized tgo3’ mRNA was distributed throughout the oocytes 4 h after injection (Fig. 7A and Table 2), in striking contrast to the tgo3’ mRNAs in transgenic oocytes, which localized to the animal pole (Fig. 2B and Table 1). Dextran with a relative molecular mass equivalent to that of tgo3’ mRNA showed a similar distribution pattern after injection into oocytes (Fig. 7B and Table 2), suggesting that the injected mRNA was not directed to localize; rather, it was passively diffused after injection. Translation of tgo3’ mRNA injected into the oocyte cytoplasm was initiated at timing much earlier than that of tgo3’ mRNA transcribed in the nucleus and transferred to the cytoplasm in transgenic zebrafish oocytes (Fig. 5G). Among 4 oocytes examined, one was activated before MIH stimulation and the others were activated precociously after MIH stimulation (Fig. 7C). These results clearly indicate that the translation of mRNAs produced in vitro and injected into oocytes was not precisely regulated in the oocytes. Therefore, nuclear events, such as transcription and splicing, are necessary to drive the specific localization in oocytes and to regulate the accurate timing of translational activation of cyclin B1 mRNA.

Discussion

In this study, we distinguished the role of cyclin B1 ORF from that of 3’UTR in localization and translational control of the mRNA by using genetic analysis in combination with real-time imaging of the translation site and timing of temporally
controlled mRNA in living cells. Our results demonstrated the following roles of the 3’UTR: 1) directing mRNA localization to the animal pole of oocytes (Fig. 3), 2) mediating translational repression in immature oocytes (Fig. 4), and 3) promoting translational activation and cytoplasmic polyadenylation during oocyte maturation (Figs. 4, 6). Furthermore, we demonstrated unexpected roles of the cyclin B1 ORF in anchoring the mRNA on the animal polar cytoplasm of full-grown oocytes (Figs. 2, 3) and determining the accurate timing of translational activation and cytoplasmic polyadenylation during oocyte maturation (Figs. 5, 6). The apparent differences in localization and translation between mRNAs transcribed in vitro and in vivo indicate that experience in the nucleus is a prerequisite for cytoplasmic regulation of the mRNA (Fig. 7).

Importance of nuclear factors in translational control as revealed by genetic analysis

Mechanisms of mRNA localization and translational control have been analyzed by injection of in vitro-synthesized mRNAs into Drosophila or Xenopus oocytes. However, it remains uncertain whether the exogenous mRNAs introduced directly into the cytoplasm were regulated by the same mechanisms as those for endogenous mRNAs that was transcribed in the nucleus and transported to the cytoplasm. Indeed, a fluorescent bicoid RNA moves on microtubules to the closest cortical surface when injected directly into the Drosophila oocyte cytoplasm, while it moves to the anterior, its final destination, when injected first into the nurse cell cytoplasm and then withdrawn and re-injected into the oocyte (Cha et al., 2001). These results indicate that cytoplasmic control of mRNAs is not solely dependent on their cis-acting elements but also dependent on a series of events in which trans-acting factors are involved; namely, key trans-acting factors must bind to their target cis-acting elements in definite order to ensure the precise spatio-temporal control of mRNA translation.

Several lines of evidence suggested that nuclear factors are involved in both localization and translational control of mRNA in the cytoplasm (see reviews, Kloc and Etkin, 2005; Palacios, 2002). Direct evidence that a machinery controlling localization and translation of mRNA in the cytoplasm is formed in the nucleus has been provided by biochemical analyses using Xenopus oocyte nuclear extracts in combination with injection of an in vitro-synthesized RNA or DNA plasmid into the oocyte nucleus (Kress et al., 2004; Lin et al., 2010). However, injection of RNA and DNA plasmid into
the oocyte nucleus is technically difficult, and this technique cannot follow a long-term process, such as oogenesis. Thus, transgenic animals that produce various reporter mRNAs through transcription in the nucleus, as in the case of endogenous mRNAs, are useful for analyzing the nuclear factors, as well as the cytoplasmic factors, involved in translational control. Although studies using *Drosophila* have shown that introduction of reporter genes into the genome is useful for elucidating the mechanisms of localization and translational control of mRNA during development and oogenesis (Forrest and Gavis, 2003; Gavis and Lehmann, 1994), analysis using transgenic individuals is still difficult to perform in vertebrates, especially in *Xenopus* having a long lifecycle. In this study, we used a genetic approach combining a vertebrate model animal, zebrafish, with *Tol2* transposon-mediated germ-line transmission, because we previously demonstrated that this system is useful for analyzing maternal factors deposited in vertebrate oocytes (Kotani and Kawakami, 2008). Using transgenic zebrafish oocytes that produce reporters for analyzing localization and translation of *cyclin B1* mRNA, we succeeded in demonstrating apparent differences between the reporter mRNAs transcribed *in vivo* (Figs. 2-6) and *in vitro* (Fig. 7); the *in vivo*-transcribed reporter, but not the *in vitro*-transcribed one, can recapitulate the localization and the precise control of translational activation of *cyclin B1* mRNA in the oocytes. It is thus confirmed that the transgenic zebrafish is useful for elucidating mechanisms of translational regulation of localized mRNAs in oocytes. In addition, the zebrafish produces a considerable number of oocytes to be analyzed biochemically and its oocyte is large enough for manipulations such as microinjection, nuclear transfer and cell graft. Given that genetic, biochemical and cell biological approaches are available for zebrafish, this model animal provides an excellent experimental system to investigate the mechanisms underlying spatio-temporal control of maternal mRNA translation during oogenesis, oocyte maturation and embryogenesis in vertebrates.

**Processes of cyclin B1 mRNA localization to the animal pole of oocytes**

The transcript of tgo3’ reporter gene was localized in the cortical cytoplasm beneath the animal pole of oocytes from stage III to full-grown stage IV (Fig. 3B, F), consistent with the localization of *cyclin B1* transcript during oogenesis (Fig. 3A, E) (Kondo et al., 2001). In contrast, the transcript of tg3’ reporter gene was localized in stage III oocytes but diffused in full-grown oocytes (Fig. 3C, G). Mechanisms by which mRNA is
localized to its final destination can be divided into two modes. One is a transporting and anchoring mode as found in ASH1 mRNA in budding yeast; ASH1 mRNA is transported by Myo4 motor protein along actin filaments and anchored at the distal tip (Takizawa et al., 1997). The other mode is a diffusion and anchoring mode as in the case of nanos mRNA in Drosophila oocytes; nanos mRNA is passively diffused throughout the cytoplasm until trapped by a localized anchor at the posterior of oocytes (Forrest and Gavis, 2003). Although the diffusion and anchoring mode is not excluded, it is likely that the transporting and anchoring mode functions in the localization of cyclin B1 mRNA in zebrafish oocytes. In this case, the mRNA is transported to the animal pole of oocytes depending on the 3’UTR sequences and stably anchored there depending on the ORF sequences. This model is consistent with our findings that the tg3’ mRNA was distributed in the hemisphere of full-grown oocytes in various patterns (Fig. 2D-F), since this phenomenon is assumed to be caused by the transport of mRNA to the animal pole through the 3’UTR and the failure in its anchoring due to the lack of ORF sequences. To verify this possibility, we need to observe the movement of reporter mRNA in living oocytes. To this end, we are currently producing transgenic zebrafish that carry reporter mRNAs containing MS2 binding sites, which can be labeled by MS2-GFP.

Visualization of temporally controlled translation of cyclin B1 mRNA

Following development of a technique for imaging the translation site using the biarsenial dyes FlAsH and ReAsH, which detect the sites of nascent polypeptide chains containing a TC-tag, Rodriguez et al. (Rodriguez et al., 2006) analyzed spatially controlled translation of β-actin mRNA in mammalian cultured cells and found that β-actin was synthesized and accumulated at the cell periphery, the final destination of the mRNA. This technique seems valuable for detecting the precise translation site of mRNAs, but temporally controlled mRNA translation has never been directly demonstrated in real time to date. We attempted to visualize the temporally controlled translation of cyclin B1 mRNA during oocyte maturation using this technique and succeeded in real-time detection of the site and time of translation of cyclin B1 mRNA (Fig. 5). This method allows us to analyze the translation of mRNAs in a single oocyte throughout the time course of the experiment, having an advantage over a common biochemical method in which several oocytes must be extracted at various time points.
during the course of the experiment to analyze polyadenylation or protein synthesis.

**Requirement of ORF sequence for temporal control of mRNA translation**

In general, localization and translational control of mRNAs are mediated by trans-acting factors that associate with cis-acting elements in the 5' or 3'UTR of target mRNAs (Groppo and Richter, 2009; Kuersten and Goodwin, 2003). Involvement of the ORF sequences in translational control was only reported in the case of spatial control of *ASH1* mRNA translation in budding yeast. During anaphase, *ASH1* mRNA localized to the distal tip of daughter cells sorts Ash1p to the daughter cell nucleus, resulting in the inhibition of mating type switching in daughter cells (Long et al., 1997; Takizawa et al., 1997). The cis-acting localization elements within the ORF of *ASH1* mRNA play a role in the regulation of its translation (Chartrand et al., 2002). A recent study has demonstrated that Khd1p, a protein with three RNA-binding hnRNP K homology domains, associates with one of the localization elements within the ORF and represses *ASH1* mRNA translation until it reaches the distal tip of daughter cells. Khd1p is phosphorylated at the distal tip by the yeast casein kinase Yck1p localized to the plasma membrane, resulting in local activation of *ASH1* mRNA translation (Paquin et al., 2007).

In contrast to *ASH1* mRNA, *cyclin B1* mRNA is localized to the cortical cytoplasm beneath the animal pole of stage III to IV oocytes and remains repressed during oogenesis and oocyte maturation until the time when Cyclin B1 protein is required. Thus, the translation of *cyclin B1* mRNA is categorized as temporally controlled. In this study, we demonstrated that the ORF sequences are involved in determining the timing of translational activation of *cyclin B1* mRNA after response to MIH. Therefore, this is the first demonstration that ORF sequences are required for temporal control of mRNA translation.

Cytoplasmic polyadenylation is known to play a key role in translational activation of mRNAs. We demonstrated in this study that *cyclin B1* mRNA is polyadenylated during zebrafish oocyte maturation by RNA ligation-coupled RT-PCR, consistent with results obtained by high-resolution RNA blot hybridization (Zhang and Sheets, 2009). Polyadenylation of *cyclin B1* mRNA is mediated by the cytoplasmic polyadenylation element-binding protein CPEB (Mendez and Richter, 2001). We previously reported that an RNA-binding protein, Pumilio, which recognizes the Pumilio-binding element (PBE) on the *cyclin B1* 3'UTR, regulates the timing of translational activation of *cyclin*
B1 mRNA during *Xenopus* oocyte maturation (Nakahata et al., 2001; Nakahata et al., 2003). We confirmed the existence of Pumilio proteins in zebrafish oocytes and the presence of PBE in the 3’UTR of zebrafish *cyclin B1* mRNA (Unpublished results), suggesting the involvement of Pumilio in regulating the timing of translational activation during zebrafish oocyte maturation. Furthermore, recent studies have demonstrated that the 3’UTR of *cyclin B1* mRNA contains binding sites of CUG-BP1 (also known as EDEN-BP), which participates in the temporal and spatial control of *xCR1* mRNA translation during *Xenopus* embryogenesis (Meijer et al., 2007; Sugimura and Lilly, 2006; Zhang et al., 2009), raising the possibility that CUG-BP1 plays a role in the translational regulation of *cyclin B1* mRNA during zebrafish oocyte maturation.

In addition to 3’UTR sequences, we demonstrated in this study that the *cyclin B1* ORF sequence is also involved in regulating the timing of cytoplasmic polyadenylation (Fig. 6), although cis-acting elements in the ORF and functionally relevant trans-acting factors remain to be identified. In this regard, it is notable that the temporal control of *histone B4* mRNA polyadenylation during *Xenopus* oocyte maturation requires not only its 3’UTR but also its ORF or 5’UTR (de Moor and Richter, 1997). Since details of these mechanisms are unknown, it is necessary to clarify whether the ORF-dependent mechanisms of *histone B4* and *cyclin B1* mRNA polyadenylation employ similar cis-acting elements and trans-acting factors or they employ completely different players, as a first step to understand molecular mechanisms of the ORF-dependent temporal control of mRNA translation.

According to our findings in this study, we propose a model of localization and translation mechanism of *cyclin B1* mRNA in oocytes. In the nucleus, *cyclin B1* transcript is recognized by trans-acting factors, some of which associate with the ORF sequences and others of which associate with the 3’UTR sequences. After the mRNA has been transported to the cytoplasm, the nucleus-derived trans-acting factors deposited on the 3’UTR drive the mRNA to the animal pole of oocytes and mediate translational repression, in collaboration with other trans-acting factors, such as Pumilio, while those deposited on the ORF mediate anchoring of the mRNA on the animal pole. In response to MIH, the trans-acting factors on the 3’UTR promote translational activation, while those on the ORF regulate the accurate timing of translational activation of *cyclin B1* mRNA cooperatively with Pumilio on the 3’UTR. Further studies are necessary to evaluate 1) what molecules associate with the *cyclin B1* ORF
sequence and 2) whether the trans-acting factors on the ORF themselves or the localization to the animal pole itself determines the accurate timing of translation.

Conclusions

We have visualized the site and time of temporally controlled translation of cyclin B1 mRNA and revealed the requirement of the ORF sequence for temporal control of translation by a genetic approach combined with real-time imaging. This method could be applied to any genes of interest. We believe that this new approach should improve our understanding of molecular mechanisms by which mRNA translation is temporally and spatially regulated.

Acknowledgements

We thank Dr. Kazuki Horikawa, National Institute of Genetics, for technical advice in detection of ReAsH fluorescence.

References


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.


Spatially restricted translation of the xCR1 mRNA in *Xenopus* embryos. Mol.
Cell. Biol. 29, 3791-3802.
### TABLE 1. Localization of the reporter mRNA in full-grown oocytes

<table>
<thead>
<tr>
<th>Localized signal</th>
<th>Transgenic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tgo3'</td>
</tr>
<tr>
<td>Aggregation</td>
<td>131/131 (100%)</td>
</tr>
<tr>
<td>Class I</td>
<td>0/131 (0%)</td>
</tr>
<tr>
<td>Class II</td>
<td>0/131 (0%)</td>
</tr>
<tr>
<td>Class III</td>
<td>0/131 (0%)</td>
</tr>
<tr>
<td>Not localized</td>
<td>0/131 (0%)</td>
</tr>
</tbody>
</table>
TABLE 2. Localization of the reporter mRNA and dextran injected into full-grown oocytes

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>Injected sample</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tgo3' mRNA</td>
<td>localized</td>
<td>not localized</td>
</tr>
<tr>
<td>1 hour</td>
<td>6/11 (55%)</td>
<td>5/11 (45%)</td>
<td></td>
</tr>
<tr>
<td>2 hours</td>
<td>4/14 (29%)</td>
<td>10/14 (71%)</td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td>5/38 (13%)</td>
<td>33/38 (87%)</td>
<td></td>
</tr>
</tbody>
</table>
Figure legends

Fig. 1. Structures of the reporter genes. The cycB1 5’UTR-TC-GFP-cycB1 ORF 3’UTR (tgo3’) gene contains cyclin B1 5’UTR, coding sequences of TC-tag (TC) and EGFP (GFP), stop codon (stop), cyclin B1 ORF and cyclin B1 3’UTR. The cycB1 5’UTR-TC-GFP-cycB1 3’UTR (tg3’) gene contains cyclin B1 5’UTR, coding sequences of TC and GFP, stop codon and cyclin B1 3’UTR. The cycB1 5’UTR-TC-GFP-SV40 3’UTR (tgs) gene contains cyclin B1 5’UTR, coding sequences of TC and GFP, stop codon and SV40 3’UTR (SV40 3’UTR). These genes are transcribed under the control of Xenopus EF1α promoter.

Fig. 2. Localization of cyclin B1 and reporter mRNAs in full-grown oocytes. (A-F) Whole-mount in situ hybridization using cyclin B1 (A) and gfp (B-F) probes of full-grown oocytes derived from transgenic fish carrying the tgo3’ (A, B), tgs (C) or tg3’ (D-F) gene. Arrows indicate localized signals. cyclin B1 and tgo3’ mRNAs was localized as an aggregation (A, B), while tgs mRNA was distributed throughout the oocytes (C). tg3’ mRNA was distributed in the hemisphere of oocytes in various patterns, Class I (D), II (E) and III (F) (see text). Bars indicate 100 µm. (G) Amount of the reporter mRNAs, normalized to that of β-actin mRNA. Real-time PCR using GFP primers showed that the transcripts of three reporter genes are deposited at similar levels in full-grown oocytes. Error bars indicate mean ± standard error of the mean (s.e.m.), n = 3.

Fig. 3. Localization of cyclin B1 and reporter mRNAs during oogenesis. (A-H) Section in situ hybridization using cyclin B1 (A, E) and gfp (B-D, F-H) probes of stage III (A-D) and stage IV (E-H) oocytes derived from wild-type (A, E) and transgenic fish carrying tgo3’ (B, F), tg3’ (C, G) or tgs (D, H) gene. Arrows indicate the micro-pile existing at the animal pole of fish oocytes. cyclin B1 and tgo3’ mRNAs were localized in the cortical cytoplasm beneath the animal pole of stage III (A, B) and IV (E, F) oocytes. tg3’ mRNA was localized to the animal pole of stage III oocytes (C) but disappeared in stage IV oocytes (G). tgs mRNA showed no localization during oogenesis (D, H). GV, germinal vesicle. Bars indicate 100 µm.

Fig. 4. Translational regulation of reporter mRNAs in immature and mature oocytes.
(A-D) Immature and mature oocytes derived from wild-type (A) and transgenic fish carrying $tgs$ (B), $tgo3'$ (C) and $tg3'$ (D) genes. Bright-field views (upper panels) and GFP fluorescence (lower panels) are shown. No GFP fluorescence was detected in wild-type immature and mature oocytes (A), while GFP fluorescence was observed in immature and mature oocytes expressing the $tgs$ mRNA (B). GFP fluorescence was not detected in immature oocytes but was visible in mature oocytes expressing the $tgo3'$ and $tg3'$ mRNA (C, D). Bars indicate 100 µm. GV, germinal vesicle.

Fig. 5. Real-time imaging of temporally controlled translation. (A) Schematic views for visualization of the translation site and timing during oocyte maturation. The *cyclin* $B1$ mRNA is localized to the animal pole of immature oocytes (blue circle). After MIH stimulation, the reporter mRNA is translated, which is detected by binding of ReAsH dye to the nascent polypeptide chains of TC-tag. The GFP protein is matured at the later stage. (B, C) Visualization of ReAsH signal in cleavage-stage embryos. Embryos derived from wild-type fish (B) and transgenic fish carrying the $tgs$ gene (C) and treated with ReAsH dye showing the fluorescent ReAsH signal (ReAsH) in embryos expressing TC-tagged GFP (GFP) but not in wild-type embryos. GFP in yolk granule (asterisk) is unable to be detected by ReAsH dye for an unknown reason. (D, E) Real-time imaging of temporally controlled translation of the $tgo3'$ mRNA (D) and $tg3'$ mRNA (E). The times after MIH stimulation are shown as standardized time $T_{GVBD}$ (see text). Arrows indicate ReAsH signals detected at the first time after MIH treatment. Translation of $tgo3'$ mRNA was first detected at the time $T_{GVBD} = 59$ at the animal pole of the oocyte. The translational site was retained at the animal pole during oocyte maturation (D). In contrast, translation of $tg3'$ mRNA was first detected at the time $T_{GVBD} = 24$ at the animal pole of the oocyte. The translational site became widely distributed throughout the hemisphere of the oocyte during oocyte maturation (E). (F) Effects of puromycin treatment on the ReAsH signal. The oocyte expressing $tgo3'$ mRNA was injected with ReAsH dye and treated with MIH. The ReAsH signal detected after MIH treatment [Puro(-)] was dispersed by puromycin treatment [Puro(+)]. (G) The times that translation of the reporter mRNA occurs after MIH stimulation. Error bars indicate mean ± s.e.m., $n = 7$ for $tgo3'$ and $n = 4$ for $tg3'$, asterisk, $P < 0.05$ (Student’s $t$-test). GV, germinal vesicle.
Fig. 6. Cytoplasmic polyadenylation of cyclin B1 and reporter mRNAs during oocyte maturation. (A) Cytoplasmic polyadenylation of cyclin B1 (cyclin B1), tgo3’ (tgo3’) and tg3’ (tg3’) mRNA in immature (Im) and mature (M) oocytes. The poly(A) tails of all mRNAs remained short in immature oocytes and were elongated in mature oocytes. The efficiency of polyadenylation of zebrafish cyclin B1 is lower than that of Xenopus cyclin B1 because of the differences in the 3’UTR sequences. Bars indicate the elongated poly(A) tail. (B) Time courses of polyadenylation of cyclin B1, tgo3’ and tg3’ mRNAs. Dotted lines indicate the basal size of the poly(A) tails. Asterisks indicate when polyadenylation of each mRNA is initiated. Polyadenylation of cyclin B1 and tgo3’ mRNAs was initiated at the time TGVBD50 = 60, while that of tg3’ mRNA was initiated at TGVBD50 = 25. (C) The times that polyadenylation of cyclin B1 and reporter mRNAs occurs after MIH stimulation. Error bars indicate mean ± s.e.m., n = 3, asterisk, P < 0.05 (Student’s t-test).

Fig. 7. Localization and translational control of in vitro-transcribed mRNA in oocytes. (A) Whole-mount gfp in situ hybridization of a full-grown oocyte fixed 4 h after injection with tgo3’ mRNA. (B) Dextran fluorescence of the full-grown oocyte 4 h after injection. The in vitro-transcribed tgo3’ mRNA and dextran appeared to be diffused after injection. (C) The times when translation of the injected mRNA occurs after MIH stimulation. Translational repression of the tgo3’ mRNA was derepressed before (TGVBD = 0) or after MIH treatment, with timings much earlier than that of the mRNA transcribed in vivo (Fig. 5G).
Figure 1

cycB1 5'UTR-TC-GFP-cycB1 ORF 3'UTR (tg3')

cycB1 5'UTR-TC-GFP-cycB1 3'UTR (tg3')

cycB1 5'UTR-TC-GFP-SV40 3'UTR (tgs)
Figure 2

A: cyclin B1  B: tgo31  C: gs
D: tgo31  E: tgo31  F: tgo31

G: Amount of transcript (GFP/β-actin)

<table>
<thead>
<tr>
<th>Transgene</th>
<th>-</th>
<th>tgo31</th>
<th>tg31</th>
<th>tgs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount</td>
<td>0.05</td>
<td>0.10</td>
<td>0.12</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Figure 3
Figure 4

A  Wild-type
   immature  mature
   ![Images of immature and mature wild-type cells with GV stage indicated]

B  tgs
   immature  mature
   ![Images of immature and mature tgs cells with GV stage indicated]

C  tgo3'
   immature  mature
   ![Images of immature and mature tgo3' cells with GV stage indicated]

D  tg3'
   immature  mature
   ![Images of immature and mature tg3' cells with GV stage indicated]
Figure 5

A) Immature cyclin B1 mRNA MIH ReAsH signal Mature GFP signal

Visualization of translation

TC-tag GFP

Ribosome

B) GFP ReAsH

C) GFP ReAsH

D) 30 39 48 59 100 122 171

E) 4 24 40 63 100 116 136

F) MIH Puromycine signal Puro(-) Puro(+)

G) Time of translational activation (TGVBD)

GFP

ReAsH

TC-tag

GFP

MIH

Puro

Reporter mRNA: tgo3' tg3'

*
Figure 6

A  

<table>
<thead>
<tr>
<th></th>
<th>cyclin B1</th>
<th>tgo3'</th>
<th>tg3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Im</td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
</tr>
<tr>
<td>M</td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
</tr>
</tbody>
</table>

B  

**cyclin B1 mRNA**

<table>
<thead>
<tr>
<th>Time after MIH stimulation (T GVBD50 )</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>(bp)</td>
<td>100</td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
</tr>
</tbody>
</table>

**tgo3' mRNA**

<table>
<thead>
<tr>
<th>Time after MIH stimulation (T GVBD50 )</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>(bp)</td>
<td>100</td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
</tr>
</tbody>
</table>

**tg3' mRNA**

<table>
<thead>
<tr>
<th>Time after MIH stimulation (T GVBD50 )</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>125</th>
</tr>
</thead>
<tbody>
<tr>
<td>(bp)</td>
<td>100</td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
<td><img src="200bp" alt="Image" /></td>
<td><img src="100bp" alt="Image" /></td>
<td><img src="300bp" alt="Image" /></td>
</tr>
</tbody>
</table>

C  

<table>
<thead>
<tr>
<th>mRNA</th>
<th>cyclin B1</th>
<th>tgo3'</th>
<th>tg3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of polyadenylation (T GVBD50 )</td>
<td>50</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
</tr>
<tr>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
</tr>
<tr>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
<td><img src="80bp" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 7

(A) Image of a control sample;
(B) Image of a sample with mRNA: tgo3;
(C) Graph showing time of translational activation (T_{GVA}) against mRNA: tgo3.