Real-time imaging of actin filaments in the zebrafish oocyte and embryo

Yumiko Nukada[1,†], Mayu Horie[1,†], Akimasa Fukui[2], Tomoya Kotani[3]* and Masakane Yamashita[3]

[1]Biosystems Science Course, Graduate School of Life Science, Hokkaido University, Sapporo 060-0810, Japan
[2]Laboratory of Tissue and Polymer Sciences, Faculty of Advanced Life Science, Hokkaido University, Sapporo 060-0810, Japan
[3]Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

†Those authors contributed equally to this work.
*Address correspondence to:
Tomoya Kotani,
Department of Biological Sciences, Faculty of Science, Hokkaido University,
North 10 West 8, Sapporo, Hokkaido 060-0810, Japan
E-mail: tkotani@sci.hokudai.ac.jp

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Abstract

Dynamic changes of cytoplasmic and cortical actin filaments drive various cellular and developmental processes. Although real-time imaging of actin filaments in living cells has been developed, imaging of actin filaments in specific cells of living organisms remains limited, particularly for analysis of gamete formation and early embryonic development. Here, we report production of transgenic zebrafish expressing the C-terminus of Moesin, an actin filament-binding protein, fused with green fluorescent protein or red fluorescent protein (GFP/RFP-MoeC), under the control of a *cyclin B1* promoter. GFP/RFP-MoeC was expressed maternally, which labels the cortical actin cytoskeleton of blastula-stage cells. High levels of GFP/RFP fluorescence were detected in the adult ovary and testis. In the ovaries, GFP/RFP-MoeC was expressed in oocytes but not in follicle cells, which allows us to clearly visualize the organization of actin filaments in different stages of the oocyte. Using full-grown oocytes, we revealed dynamic changes of actin columns assembled in the cortical cytoplasm during oocyte maturation. The number of columns slightly decreased in the early period before germinal vesicle breakdown (GVBD) and then significantly decreased at GVBD, followed by recovery after GVBD. Our transgenic fish are useful for analyzing dynamics of actin filaments in oogenesis and early embryogenesis.

Introduction

Polymerization and depolymerization of actin protein play fundamental roles in cellular and developmental processes including cell shape changes, intracellular signal transduction, and cell locomotion. To elucidate the dynamics of actin filaments, techniques for real-time imaging of the protein in cultured, living cells have been developed [Ballestrem et al., 1998; Doyle and Botstein, 1996; Gerisch et al., 1995; Kreis et al., 1982; Westphal et al., 1997]. Dynamic changes of cell shapes in embryos and adult tissues were first visualized in *Drosophila* [Edwards et al., 1997] by labeling the actin cytoskeleton with green fluorescent protein (GFP) fused with an actin-binding domain of Moesin, one of the Ezrin/Radixin/Moesin (ERM) family proteins acting as a membrane-cytoskeleton linker. ERM proteins contain an FERM (4.1 protein, Ezrin, Radixin, Moesin) domain responsible for binding to the plasma membrane at the N-terminal region [Algrain et al., 1993; Chishti et al., 1998] and an actin-binding domain at the C-terminal tail [Turunen et al., 1994]. GFP fused with the 140-amino-acid C-terminal tail of Moesin containing the actin-binding domain was able to label the cortical actin cytoskeleton in cells of *Drosophila* embryos and adult tissues without affecting structures and dynamics of the cell cytoskeleton and embryonic development when expressed in whole embryos under control of the heat shock protein promoter [Edwards et al., 1997] and in specific tissues under control of the Gal4-UAS system [Dutta et al., 2002].

Because of external development and transparency of the embryo, zebrafish has been utilized for live imaging of cell organelles and cytoskeletons to analyze the cellular processes during vertebrate development [Asakawa and Kawakami, 2010; Distel et al., 2010]. In a recent study, the actin cytoskeleton of endodermal cells was visualized during embryonic development by using GFP fused with the calponin homology domain of Utrophin, an actin-binding protein, which was expressed under control of the *sox17* promoter [Woo et al., 2012]. However, there are still limitations for visualizing the dynamics of actin filaments in specific types of cells in vertebrates, particularly oocytes and sperm cells.

Full-grown vertebrate oocytes arrest meiotic cell cycle at prophase I and deposit dormant mRNAs required for progression of meiosis and embryogenesis. We previously showed that assembly of actin filaments in the full-grown zebrafish oocyte is required for the formation of RNA granules containing dormant *cyclin B1* mRNA at the animal

polar cytoplasm [Kotani et al., 2013]. Translational activation of *cyclin B1* mRNA occurs after induction of oocyte maturation, a process in which oocytes resume meiotic cell cycle and subsequently arrest at metaphase II. The accurate timing of translational activation of *cyclin B1* mRNA is important for normal progression of maturation processes [Davydenko et al., 2013; Kondo et al., 2001; Kotani and Yamashita, 2002; Kotani et al., 2013]. Depolymerization of the filaments by treatment with the actin-depolymerizing drug cytochalasin B promotes disassembly of the granules and precocious translational activation of the RNA, suggesting a link between actin assembly and translational control of *cyclin B1* mRNA [Kotani et al., 2013]. Phalloidin staining of full-grown immature oocytes showed the presence of dense actin networks in the cortical cytoplasm, which disappeared after completion of oocyte maturation [Kondo et al., 2001]. However, dynamics of actin filaments in oocytes remains completely unresolved.

Here, we describe the production of transgenic zebrafish expressing GFP and red fluorescent protein (RFP) fused with the Moesin C-terminal tail (MoeC) under the control of a *cyclin B1* promoter. GFP/RFP-MoeC was expressed in oocytes from early to late stages (stages I-V), but not in follicle cells surrounding the oocytes, clearly visualizing the organization of actin filaments in different stages of oocytes. Actin columns that formed in the cortical cytoplasm of full-grown oocytes were partially depolymerized in the early period after induction of oocyte maturation, being consistent with the timing of translational activation of *cyclin B1* mRNA. Our results support a link between actin dynamics and translational control of *cyclin B1* mRNA and indicate that the transgenic zebrafish is useful for analyzing actin dynamics in oogenesis.

Results

Expression of GFP/RFP-MoeC under the control of a *cyclin B1* promoter

The zebrafish Moesin contains an FERM domain at the N-terminal region and an actin-binding domain at the C-terminal tail (Fig. 1A). To effectively label actin filaments, GFP or RFP was fused with the Moesin C-terminal tail (382-579 amino acids) including the actin-binding domain (Fig. 1A; GFP/RFP-MoeC), as in the case of live imaging in *Drosophila* [Dutta et al., 2002; Edwards et al., 1997]. In order to visualize structures and dynamics of actin filaments in the oocyte and embryo during oogenesis and early development, the sequences encoding GFP/RFP-MoeC was placed

downstream of a promoter of the *cyclin B1* gene (Fig. 1B). Since the transcript of *cyclin B1* gene is abundantly expressed in oocytes from early to late stages (stages I-V) but is unexpressed in follicle cells surrounding the oocytes [Kondo et al., 2001], we expected that the *cyclin B1* promoter could induce expression of GFP/RFP-MoeC in oocytes from early to late stages but not in follicle cells, which allows us to clearly visualize organization of actin filaments in oocytes throughout oogenesis. These constructs were transmitted into germ cells by using the *Tol2* transposon system [Kotani et al., 2006].

Zygotic expression of GFP-MoeC was first detected weakly in the whole body and strongly in the eye at 14 hours post-fertilization (hpf) (Fig. 1C). Essentially the same expression pattern was observed in embryos at 24 hpf (Fig. 1D). Bright fluorescence of maternally expressed GFP-MoeC was detected from the one-cell stage to 24 hpf (Fig. 1E-G). Although zygotic RFP-MoeC expression was undetectable probably due to low brightness of monomeric RFP [Campbell et al., 2002], its maternal expression was observed from the one-cell stage to the epiboly stage (Fig. 1H-J). Maternally expressed GFP-MoeC was distributed at the cortical cytoskeleton of blastula-stage cells (Fig. 1K). Phalloidin staining of the embryos demonstrated that structures of the cortical actin cytoskeleton were labeled in detail with GFP-MoeC (Fig. 1L).

Fluorescence of GFP/RFP-MoeC in the adult fish was detected by a fluorescence stereomicroscope in ovaries and testes but not in other organs (Fig. 2A-C). As expected, the *cyclin B1* promoter recapitulated the expression pattern of the *cyclin B1* transcript in the ovary; i.e., bright GFP/RFP fluorescence was observed in oocytes from early to late stages (Fig. 2D and Supporting Information Fig. S1). We focused on GFP/RFP-MoeC expression in the ovary in this study, and its expression in the testis will be reported elsewhere. Confocal micrographs of the ovaries stained with phalloidin demonstrated that GFP-MoeC was strictly expressed in the oocytes but not in follicle cells surrounding the oocytes and that dense actin networks in the oocyte cortical cytoplasm were clearly and strictly labeled by GFP-MoeC (Fig. 2E and F).

Organization of actin filament in oocytes from early to late stages

Restricted expression of GFP/RFP-MoeC in the oocytes allows us to discriminate the organization of actin filaments in oocytes from that in follicle cells. This is extremely difficult in the ovary stained with phalloidin because of the tight association of follicle cells with oocytes (Fig. 3A and B). To characterize the actin organization in different

stages of oocytes in detail, we observed live ovaries derived from *Tg*(*ccnb1*:GFP-MoeC) females under the confocal microscope. Thin actin cytoskeletons were observed at the cortical region of stage I to II oocytes (Fig. 3C and D). The cortical actin filaments became broadly distributed in the subcortical regions of stage III to IV oocytes (Fig. 3E and F). The changes in the organization of actin filaments would be related to deposition of yolk granules in oocytes, which begins from stage III [Selman et al., 1993].

Dynamics of the actin columns after induction of oocyte maturation

Zebrafish oocytes deposit the translationally repressed *cyclin B1* mRNA as a large number of RNA granules in the animal polar cytoplasm beneath a micropyle thorough which the sperm fertilizes the egg (Fig. 4A) [Kotani et al., 2013]. After induction of oocyte maturation by treatment with a maturation-inducing hormone (MIH), these RNA granules were disassembled in individual oocytes halfway between MIH addition and GVBD (Fig. 4B and C), consistent with the timing of translational activation of the RNA [Kotani et al., 2013]. Intriguingly, depolymerization of actin filaments induces dissociation of the RNA granules, and, conversely, stabilization of the actin filaments prevents the disassembly of RNA granules even in oocytes treated with MIH, indicating that the granule disassembly is dependent on depolymerization of actin filaments [Kotani et al., 2013]. These results suggested that the actin filaments assembled in the animal polar cytoplasm would be depolymerized prior to the disassembly of RNA granules.

To assess this possibility, we analyzed the dynamics of actin networks in the animal polar cytoplasm after induction of oocyte maturation. First, the 3-dimensional structures of actin filaments were reconstructed by scanning z-stack images of the animal polar cytoplasm beneath a micropyle (Fig. 4D). Interestingly, many straight actin columns were organized across the cortical cytoplasm in the inner to outer direction and thereby each column was formed in parallel (Fig. 4E). The length of columns was 13.2 ± 2.0 μm (n = 36), consistent with the layer of *cyclin B1* mRNA distribution in the cytoplasm (Fig. 4A) [Kotani et al., 2013]. Though the detailed structure of actin filaments was not observed by immunofluorescence of the oocyte sections, GFP-MoeC signal was detected within the cytoplasmic layer almost similar to that of *cyclin B1* mRNA distribution (Fig. 4F), showing that the length of actin columns (13.2 μm) does not

reflect the depth of signals detectable by the confocal microscope. In addition, live imaging of oocytes by multi-photon confocal microscopy showed the assembly of actin filaments throughout the cortical cytoplasm (Fig. 4G), indicating that the actin filaments are not specifically assembled in the animal polar cytoplasm but are broadly distributed in the oocyte cortical cytoplasm.

The actin columns at the animal polar cytoplasm were then visualized by scanning z-stack images at intervals of 5 min after induction of oocyte maturation. To quantify the changes of actin columns, the number of spots of actin columns in 100 μm^2 of cytoplasm was counted in three different regions using the z-plane 6 μm below the cortical surface, which shows the middle region of columns (Fig. 4H and I). We could not count the columns immediately after treatment of oocytes with MIH, because MIH treatment affected oocyte rotation and it took several minutes to manually correct the animal-vegetal direction of oocytes by forceps and to find a micropyle by scanning z-stack images. The number of columns was 31.7 ± 2.3 at 10 min after induction of oocyte maturation and was decreased at 20 min (23.3 ± 0.6, $p < 0.05$, Student's *t*-test). Statistical differences in the number of columns were shown from 20 to 50 min (Fig. 4J, asterisks) but not from 55 to 65 min. A significant decrease in the number of columns was observed at 70 min (10.7 ± 0.6, $p < 0.01$, Student's *t*-test), at which time the oocyte underwent GVBD. The number of columns had recovered at 75 min (25.0 ± 1.0) and maintained until 95 min after induction of oocyte maturation (Fig. 4I and J). Similar observation was demonstrated at the z-plane 3 μm below the cortical surface (Supporting Information Fig. S2A). We further obtained equivalent results using two other oocytes derived from distinct females (Supporting Information Fig. S2B and C). In contrast, no change was observed in control oocytes treated with the vehicle ethanol. These results suggest that depolymerization of actin columns (20 min after induction of maturation) precedes the disassembly of RNA granules, which has been shown to occur halfway between MIH addition and GVBD (35 min after induction of maturation in this case) [Kotani et al., 2013].

Dynamics of the individual actin columns

To analyze dynamics of individual actin columns, we observed xz-planes of actin columns in the animal polar cytoplasm at intervals of 6.5 seconds after induction of oocyte maturation. Intriguingly, individual actin columns were rapidly depolymerized

within 30-60 seconds (Fig. 5A, white arrows), and the certain numbers of columns were polymerized even after the columns were depolymerized (Fig. 5B, yellow arrows). In contrast, no change was observed in non-treated oocytes. These results show that the individual actin columns are rapidly and robustly remodeled and the total number of columns gradually decreases after induction of oocyte maturation.

We finally analyzed the effect of cytochalasin B on the columns by observing xz-planes. The actin columns were rapidly depolymerized between 5 to 10 min after treatment with 1 μg/ml of cytochalasin B (Fig. 5C). This treatment did not completely prevent polymerization of the actin columns, because the columns were polymerized even 10 min after treatment (Fig. 5C). Then, almost all columns were depolymerized 40 min after treatment (Fig. 5C). In these oocytes, the *cyclin B1* RNA granules were dispersed throughout the oocyte cytoplasm (Fig. 5D), also suggesting that the granule formation is dependent on actin filaments [Kotani et al., 2013].

Discussion

Visualization of actin filaments in oocytes during oogenesis

Analysis of actin filament organization in vertebrate oocytes has been extremely difficult because of the presence of follicle cells, which tightly surround the oocytes. To overcome this problem, we utilized the *cyclin B1* promoter to specifically express fluorescent proteins in oocytes from the beginning of oogenesis until oocytes are fully grown. Prior to using the *cyclin B1* promoter, we produced transgenic fish carrying the GFP gene downstream of the *vasa* promoter, which promotes gene expression in germline cells [Krovel and Olsen, 2002]. In these fish, GFP fluorescence was specifically detected in early-stage oocytes (stage I) but was undetectable in oocytes at a later stage (T. Kotani, unpublished data). In contrast, transgenic fish carrying the GFP gene downstream of the Ef1α promoter [Kawakami et al., 2004] showed GFP fluorescence in oocytes from early to late stages (stages I-V) and also in almost all follicle cells (T. Kotani, unpublished data). Therefore, specific expression of fluorescent proteins in oocytes from early to late stages was achieved for the first time by using the *cyclin B1* promoter.

Visualization of actin filaments in oocytes was also achieved by using the C-terminal tail of Moesin. Staining of embryos and oocytes derived from transgenic fish with phalloidin showed that detailed structures of actin filaments were clearly labeled by

GFP-MoeC (Figs. 1L and 2E, F). Since all transgenic fish were fertile and embryos derived from the fish showed normal development, expression of GFP and RFP fused with the C-terminal tail of Moesin appears to have no or minimal effect on gamete formation and embryonic development similar to the case of *Drosophila* [Dutta et al., 2002; Edwards et al., 1997]. Taken together, the results indicate that our transgenic fish are useful for analyzing the organization and dynamics of actin filaments in oocytes throughout oogenesis in addition to those in embryonic cells during early development.

GFP- and RFP-fused MoeC can be used for visualization of actin filaments simultaneously with different molecules or events in the same cell. For example, the *Tg*(*ccnb1*:GFP-MoeC) line could be used for visualization of actin filaments and translational activation of *cyclin B1* mRNA, which is detected by ReAsH fluorescence (Yasuda et al., 2010). Similarly, the *Tg*(*ccnb1*:RFP-MoeC) line could be used for visualization of actin filaments and *cyclin B1* mRNA molecules, which is labeled by the GFP-fused bacteriophage MS2 coat protein (T. Kotani, unpublished data) [Buxbaum et al., 2015].

In this study, we found that oocytes in the early stages (stages I and II) form the thin cortical actin filaments (Fig. 3C and D). This structure was not found in oocytes in the late stages (stage III and IV), and instead actin filaments were distributed broadly in the subcortical region (Fig. 3E and F). These observations suggest that the organization of actin filaments is modified at the beginning vitellogenesis. Since the zebrafish oocyte deposits yolk granules at the center of the oocyte, the oocyte cytoplasm becomes restricted in the subcortical region [Selman et al., 1993]. Our observations suggest that there is a relationship between cytoplasmic restriction and actin filament modification in the oocyte subcortical region. Although the mechanisms by which actin filaments are remodeled during zebrafish oocyte development remain unknown, actin-nucleation factors such as spire and formin proteins, which assemble the cytoplasmic actin filaments in full-grown mouse oocytes [Azoury et al., 2008; Pfender et al., 2011; Schuh and Ellenberg, 2008], might be involved in this process.

Linkage of actin filaments with translational control of RNA

In the *Drosophila* oocyte, actin filaments have been shown to play an important role in localization of *oskar* mRNA at the posterior pole, which is essential for posterior patterning in embryogenesis, because disruption of the actin assembly resulted in failure

of anchoring *oskar* mRNA at the posterior cortex [Babu et al., 2004; Cha et al., 2002]. Although these studies demonstrated the significance of actin filaments in mRNA localization, their roles in mRNA translation remain elusive. In the zebrafish oocyte, *cyclin B1* mRNA is localized at the animal polar cytoplasm, and our previous study demonstrated the presence of a link between actin assembly and translational control of *cyclin B1* mRNA, which is mediated by RNA granule formation [Kotani et al., 2013].

In this study, we showed the broad assembly of actin filaments in the zebrafish oocyte cytoplasm (Fig. 4), suggesting that the actin filaments at the animal polar cytoplasm is not involved in localization of *cyclin B1* mRNA in this region. Transportation of *cyclin B1* mRNA to the animal polar cytoplasm might be dependent on microtubules, and the actin columns would be involved in the mRNA anchoring after transported. However, we could not rule out a possibility that the detailed organization and composition of actin columns assembled in the animal polar cytoplasm are different from those assembled in other regions, resulting in accumulation of the *cyclin B1* mRNA at the animal polar region. We also showed that treatment of oocytes with cytochalasin B caused rapid and robust depolymerization of actin columns and dispersion of *cyclin B1* RNA granules (Fig. 5C and D). These results support the notion that the actin columns assembled in the animal polar cytoplasm anchor the *cyclin B1* mRNA as granules. Since the disruption of actin assembly promotes precocious translational activation of *cyclin B1* mRNA after induction of oocyte maturation, while stabilization prevents translational activation [Kotani et al., 2013], the actin filaments may indirectly protect the mRNA from accession of translational machinery by anchoring them as granules. Real-time imaging of actin dynamics revealed a rapid and robust remodeling of individual columns and a gradual decrease in the total number of actin columns in the early period after induction of oocyte maturation (Figs. 4 and 5), which precedes the translational activation of *cyclin B1* mRNA [Kotani et al., 2013; Yasuda et al., 2010]. Therefore, our observations support the link between actin assembly and translational control of the mRNA. To evaluate the linkage of actin filaments with translational control of the mRNA, we are currently producing transgenic fish that simultaneously visualizes actin filaments, *cyclin B1* RNA granules and translational activation of the mRNA in live oocytes.

Dynamic changes of actin filaments during oocyte maturation

In mouse oocytes, changes in actin networks from 30 min before GVBD (approximately 1-2 hr after resumption of meiosis) to 8 hr after GVBD, at the time of extrusion of a polar body, have been demonstrated by injection of full-grown oocytes with mRNA encoding GFP fused with the calponin homology domain of Utrophin [Azoury et al., 2011; Azoury et al., 2008; Schuh and Ellenberg, 2008]. Many of the dense networks assembled within the oocyte cytoplasm disappeared 15 min before GVBD, followed by re-formation of actin networks during subsequent bipolar spindle formation. This actin filament remodeling after resumption of meiosis is essential for polar body extrusion in the mouse oocyte [Azoury et al., 2011]. Real-time imaging of actin filaments in the zebrafish oocyte also showed a significant decrease in the number of actin columns at GVBD and quick recovery after GVBD (Fig. 4). This is consistent with the results in mouse oocyte maturation, suggesting importance of this remodeling for polar body extrusion in zebrafish oocytes. The disassembly of actin networks at GVBD is accompanied by degradation of Formin-2, an actin nucleation factor, in this period in mouse oocytes [Azoury et al., 2011]. However, the mechanism of this remodeling in zebrafish oocytes remains unknown. Identification of factors that regulate the organization of actin filaments, including those involved in remodeling of actin filaments in the early period of oocyte maturation, is an important issue to be resolved in the future.

We focused on the link between remodeling of actin filaments and translational control of mRNA at the initiation of oocyte maturation in this study. Dynamic changes of actin filaments in this period may participate in other events, such as movement of germinal vesicle toward the oocyte surface [Kotani and Yamashita, 2002]. In ovulated, unfertilized eggs, thin actin filaments are assembled at the restricted region of egg surface beneath a micropyle [Hart et al., 1992]. These filaments surround the sperm nucleus within several min after fertilization, which would promote incorporation of the nucleus into the egg cytoplasm. In addition, in the short period within 5 min after fertilization, actin filaments are broadly assembled in the egg cortical layer [Becker and Hart, 1999]. These actin filaments colocalize with non-muscle myosin II. The assembly and subsequent disassembly of actin filaments are important for the regulation of cortical granule exocytosis [Becker and Hart, 1999]. Zebrafish eggs resume meiotic cell cycle, which is arrested at metaphase II, after fertilization, followed by the cleavage

cycles. In the period of cleavage stages, assembly of actin filaments with myosin II at cleavage furrows is prerequisite for formation of cleavage planes during cytokinesis [Urven et al., 2006]. Live imaging using eggs derived from our transgenic fish will contribute to analyze dynamics of actin filaments in these processes.

Materials and methods

Plasmid construction

Sequences encoding the C-terminus of zebrafish Moesin (MoeC; 382-579 amino acids) were amplified by RT-PCR with cDNAs from an ovary and a primer set of 5'-GTCGACAAGCTCAGGAGGAGGCTGAGCGT-3' (underline indicating *Sal*I site) and 5'-TCTAGA**TCGAT**TTACATGCACTCAAACTCGTCGATTC-3' (underline indicating *Xba*I site and bold characters indicating *Cla*I site). After being cloned into pGEM-T, the amplified sequence was digested with *Sal*I and *Xba*I and ligated between the *Xho*I and *Xba*I sites of pAcGFP1-C1 or pDsRed-Monomer-C1 (Clontech). Sequences encoding GFP/RFP-MoeC were digested with *Age*I and *Cla*I and ligated between the *Age*I and *Cla*I sites of T2KXIGΔin [Urasaki et al., 2006]. Sequences from 1317 bp upstream to 15 bp downstream of the transcription initiation start site of the *cyclin B1* gene were amplified by PCR with genomic DNA from zebrafish embryos and a primer set of 5'-CTCGAGCAAAATCCCCCAATCACTATAAAA-3' (underline indicating *Xho*I site) and 5'-GGATCCAAATTGTCGCTCTTTCAAATATGATTCG-3' (underline indicating *BamH*I site). Although a transgenic zebrafish expressing GFP under the control of a *cyclin B1* promoter was reported previously [Kassen et al., 2008], we could not amplify the sequences with the primer set shown in that report for an unknown reason. Thus, we amplified the sequences upstream of the *cyclin B1* gene according to the BAC information (DKEY-267K7). After being cloned into pGEM-T, the amplified sequence was digested with *Xho*I and *BamH*I and ligated between the *Xho*I and *BamH*I sites of T2KXIGΔin containing GFP/RFP-MoeC, resulting in T2ccnb1P-GFP-MoeC and T2ccnb1P-RFP-MoeC.

Production of transgenic zebrafish

All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University. Transgenic zebrafish were generated by using *Tol2* transposon-mediated germline transmission [Kotani et al., 2006]. One nano-liter of

a solution containing 250 μg/ml of T2ccnb1P-GFP-MoeC or T2ccnb1P-RFP-MoeC construct and 250 μg/ml of in vitro-synthesized mRNAs encoding *Tol2* transposase was injected into one-cell stage embryos. The injected embryos were raised and crossed with wild-type fish. F1 embryos carrying the GFP-MoeC construct were screened by GFP fluorescence under an M165FC fluorescence stereomicroscope (Leica). Since we could not detect RFP fluorescence during embryonic development, F1 embryos carrying the RFP-MoeC construct were screened by PCR with genomic DNAs extracted from the embryos and a primer set of 5'-AACACCGAGGACGTCATCAAGGAG-3' (RFP-forward) and 5'-CTTGGAGTCCACGTAGTGGTTGCC-3' (RFP-reverse). Five lines carrying the GFP-MoeC construct, *Tg*(*ccnb1*:GFP-MoeC), and two lines carrying the RFP-MoeC construct, *Tg*(*ccnb1*:RFP-MoeC) were produced and analyzed. All lines carrying the same construct gave equivalent results.

Preparation of ovaries and testes

Ovaries and testes were dissected from adult transgenic fish in zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM Hepes, pH 7.2) and observed under an M165FC fluorescence stereomicroscope (Leica), an LSM 5 LIVE confocal microscope (Zeiss), a Fluoview FV1000MPE confocal microscope (Olympus) and a TCS SP5 confocal microscope (Leica). The oocytes were classified into five stages according to their diameters (stage I, 7-140 μm; stage II, 140-340 μm; stage III, 340-690 μm; stage IV, 690-730 μm) and meiotic stages (stage I-IV, arrested at prophase of meiosis I; stage V, resumed meiosis and arrested at metaphase of meiosis II) (Selman et al., 1993).

Phalloidin staining

Structures of actin filaments were confirmed with phalloidin. Embryos and ovaries derived from transgenic fish were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) overnight at 4˚C. After washing three times with PBS, the samples were stained with phalloidin-Alexa546 (1/100 dilution; Molecular Probes) for 30 min at 4˚C. The samples were washed three times with PBS, incubated with 10 μg/ml Hoechst 33258 for 10 min, and observed under the LSM 5 LIVE confocal microscope.

Induction of oocyte maturation

Oocytes were manually isolated from ovaries with forceps under a dissecting microscope. Oocyte maturation was induced by treatment with 1 μg/ml 17α,20β-dihydroxy-4-pregnen-3-one, an MIH in fish. The oocytes were observed under the LSM 5 LIVE confocal microscope and the TCS SP5 confocal microscope. The xz-plane images were obtained using the TCS SP5 confocal microscope by scanning y-stack images at intervals of 0.2 μm in the 1.6 μm range.

In situ hybridization and immunofluorescence

In situ hybridization with the TSA system (PerkinElmer) was performed according to the procedure reported previously [Kotani et al., 2013]. Immunofluorescence with anti-GFP antibody (Roche) was performed according to the procedure reported previously [Kotani and Yamashita, 2002]. The samples were observed under the LSM 5 LIVE confocal microscope.

Image analysis

The images were compiled using Photoshop CS5 software (Adobe). Three-dimensional structures of the actin filaments were reconstructed by using z-stack images and IMARIS software (Bitplane). The length of actin columns was measured on the 3-dimensional images. The number of actin columns was quantified using IMARIS, which enables detection of column spots according to the size (>0.2 μm) and the intensity at the center of spots.

Cytochalasin B treatment

Oocytes were treated with 1 μg/ml cytochalasin B (Sigma-Aldrich) and observed under the TCS SP5 confocal microscope. After treatment with cytochalasin B for 3 h, the oocytes were fixed with 4% paraformaldehyde in PBS and used for in situ hybridization analysis.

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Figure Legends

Fig. 1. Transgenic fish expressing GFP-MoeC or RFP-MoeC under control of the *cyclin B1* promoter. (**A**) Schematic diagrams of zebrafish Moesin (Moesin) and GFP and RFP fused with the C-terminal tail of Moesin (GFP-MoeC and RFP-MoeC). FERM, 4.1 protein, Ezrin, Radixin, Moesin domain; AB, actin-binding domain. (**B**) Schematic diagrams of the reporter genes. Sequences encoding GFP-MoeC and RFP-MoeC were placed downstream of the *cyclin B1* promoter (*cyclin B1*-P) and upstream of the polyA tail of the SV40 gene (polyA). (**C**-**D**) Zygotic expression of GFP-MoeC in embryos derived from *Tg*(*ccnb1*:GFP-MoeC) males at 14 hpf (C) and 24 hpf (D). (**E**-**G**) Maternal expression of GFP-MoeC in embryos derived from *Tg*(*ccnb1*:GFP-MoeC) females at 1-cell (E), 4-cell (F) and 80% epiboly (G) stages. (**H**-**I**) Maternal expression of RFP-MoeC in embryos derived from *Tg*(*ccnb1*:RFP-MoeC) females at 1-cell (H), 4-cell (I) and 80% epiboly (J) stages. (**K**) Confocal micrograph of GFP-MoeC in blastula cells of an embryo derived from a *Tg*(*ccnb1*:GFP-MoeC) female. (**L**) Distribution of GFP-MoeC in blastula cells. The fixed embryo was stained with phalloidin-Alexa546 (Phalloidin). A merged view is shown (Merge). Bars, 10 μm.

Fig. 2. Expression and distribution of GFP-MoeC in transgenic adult fish. (**A**) Detection of GFP fluorescence in adult fish under the fluorescence stereomicroscope. GFP was observed in *Tg*(*ccnb1*:GFP-MoeC) females but not in wild-type females (Wt) at the position of ovary. (**B**) Expression of GFP fluorescence in *Tg*(*ccnb1*:GFP-MoeC) female (upper) and male (lower) organs. Right panels show the female fish after removal of ovary (-Ovary) and the male fish after removal of testis (-Testis). GFP was observed in ovaries and testes but not in other organs. Note that gut shows auto-fluorescence (asterisks). (**C**) Expression of GFP-MoeC in the ovary and testis of *Tg*(*ccnb1*:GFP-MoeC) adult fish. (**D**) Expression of GFP-MoeC in different stages of oocytes (I-IV). (**E**) Expression and distribution of GFP-MoeC in the follicle consisting of a full-grown oocyte (stage IV). Fixed ovaries were stained with phalloidin-Alexa546 (Phalloidin) and Hoechst 33258 (Hoechst) and observed under the confocal microscope. A merged view is shown (Merge). (**F**) Enlarged views of the boxed regions in E. fc, follicle cells; oc, oocyte cytoplasm. Actin filaments assembled in the oocyte cytoplasm, but not those in follicle cells, were labeled with GFP-MoeC because of specific expression in oocytes. Bars, 2 mm in (A-B), 1 mm in (C), 100 μm in (D-E), 20 μm in

(F).

Fig. 3. Live images of actin filaments in different stages of oocytes. (**A**-**B**) Images of ovaries fixed and stained with phalloidin-Alexa546 (Phalloidin) and Hoechst 33258 (Hoechst). Merged views are shown (Merge). Follicles consisting of oocytes in stage II (A) and stage III (B). fc, follicle cells. (**C**-**F**) Live images of actin filaments in oocytes in stage I (C), II (D), III (E), and IV (F). GV, germinal vesicle; yg, yolk granules. Bars, 20 μm.

Fig. 4. Real-time images of actin filaments after induction of oocyte maturation. (**A**) Fluorescent in situ hybridization of *cyclin B1* mRNA (green) in full-grown oocytes (stage IV). *cyclin B1* mRNA is localized as a large number of granules at the animal polar cytoplasm beneath a micropyle (mp). DNA is shown in blue. The chorion is outlined by broken lines. fc, follicle cells; ch, chorion. (**B**-**C**) Disassembly of the *cyclin B1* RNA granules after induction of oocyte maturation. (B) Dense *cyclin B1* RNA granules were maintained in oocytes 30 min after induction of maturation (30 m) but had disappeared at 60 min (60 m). (C) The number of RNA granules per 100 μm^2 in individual oocytes was counted and categorized as dense (21-40), partially disassembled (2-20), and disassembled (0-1). The numbers in parentheses indicate the total number of oocytes analyzed. Time course of GVBD is shown in red. (**D**) Schematic view of scanning z-stack images of actin filaments assembled in the animal polar cytoplasm beneath a micropyle. fo, full-grown oocyte; ch, chorion; mp, micropyle. (**E**) Structures of actin filaments in the oocyte cytoplasm. Many straight columns were formed across the cortical cytoplasm in the inner (bottom) to outer (top) direction. (**F**) Immunofluorescence of GFP-MoeC in the oocyte sections. (**G**) A live image of GFP-MoeC in the full-grown oocyte using the multi-photon confocal microscopy. The cortical cytoplasmic layer exists outside of broken lines. Large yolk granules (ly) accumulated in the deep layer of oocytes show auto-fluorescence. GV, germinal vesicle. (**H**-**J**) Dynamic changes of actin columns after induction of oocyte maturation. (H) Z-stack image 6 μm below the cortical surface. (I) Time course of z-stack images of actin columns after induction of oocyte maturation. (J) The number of actin columns was counted in 100 μm^2 of the cytoplasm at three different regions indicated as boxed regions in H (means ± standard deviations). Student's *t*-test relative to the point at 10

min: *P < 0.05, **P < 0.01. Bars, 100 μm in (G), 10 μm in (A, E, F and H), 5 μm in (B and I).

Fig. 5. Real-time images of individual actin columns. (**A-B**) Time course of xz-plane images of actin columns after induction of oocyte maturation. White arrows in (A) indicate tails of the columns depolymerized in the period from 91 to 156 seconds (s), which is counted 50 min after induction of oocyte maturation. Yellow arrows in (B) indicate tails of the columns polymerized in the period from 273 to 338 seconds. Individual columns were rapidly remodeled within 30-60 seconds. (**C**) Time course of xz-plane images of actin columns after treatment of oocytes with cytochalasin B. The columns were robustly but not completely depolymerized between 5 to 10 min after cytochalasin B treatment and were almost completely depolymerized at 40 min. (**D**) Fluorescent in situ hybridization of *cyclin B1* mRNA (green) in oocytes treated with DMSO (left) and cytochalasin B (right). *cyclin B1* RNA granules were dispersed within the cortical cytoplasm in the oocytes treated with cytochalasin B. Inserts are enlarged views of the boxed regions. DNA is shown in blue. The chorion is outlined by broken lines. fc, follicle cells; ch, chorion; mp, micropyle. Bars, 2 μm in (A-C), 5 μm in (D).