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Role of Mos/MEK/ERK cascade and Cdk1 in Ca^{2+} oscillations in fertilized ascidian eggs

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ABSTRACT

Intracellular calcium ion concentration ($[Ca^{2+}]_i$) transients are observed in the fertilized eggs of all species investigated so far, and are critical for initiating several events related to egg activation and cell cycle control. Here, we investigated the role of the Mos/MEK/ERK cascade and Cdk1 on Ca^{2+} oscillations in fertilized ascidian eggs. The egg of the ascidian *Phallusia nigra* shows $[Ca^{2+}]_i$ oscillations after fertilization: Ca^{2+} waves immediately following fertilization (phase I), and $[Ca^{2+}]_i$ oscillations between the first and second polar body extrusions (phase II). Our results show that in *P. nigra* eggs, ERK activity peaked just before the extrusion of the first polar body, and decreased gradually, eventually disappearing at the extrusion of the second polar body. Cyclin-dependent protein kinase 1(Cdk1) activity decreased to undetectable levels immediately after fertilization, and then periodically increased according to the meiotic and mitotic cell cycle. When the unfertilized eggs were incubated with U0126, an inhibitor of MEK, before insemination, ERK was immediately inactivated, and the phase II $[Ca^{2+}]_i$ oscillations disappeared. Alternatively, when the constitutively active Mos protein (GST-Mos) was injected into the unfertilized eggs, ERK activity was preserved for at least 120 min after fertilization, and the phase II $[Ca^{2+}]_i$ oscillations lasted for more than120 min after the second polar body extrusion. These results suggest that ERK activity is necessary for maintaining $[Ca^{2+}]_i$ oscillations. GST-ΔN85-cyclin, which maintains Cdk1 activity, caused ERK activity in the eggs to persist for over 120 min after fertilization, and prolonged $[Ca^{2+}]_i$ oscillations. Moreover, the effects of GST-ΔN85-cyclin on the egg were abrogated by the application of U0126. Thus, Cdk1-mediated $[Ca^{2+}]_i$ oscillations seem to require ERK activity. However, GST-Mos triggered $[Ca^{2+}]_i$ oscillations after the second polar body extrusion, whereas GST-ΔN85-cyclin did not, although it prolongs the duration of $[Ca^{2+}]_i$ oscillations. Interestingly, GST-ΔN85-cyclin increased the frequency of $[Ca^{2+}]_i$ transients in the Mos-induced $[Ca^{2+}]_i$ oscillations after the extrusion of the second polar body. Thus, Cdk1 could maintain, but not activate, ERK and $[Ca^{2+}]_i$ oscillations. ERK activity and $[Ca^{2+}]_i$ oscillations seem to form a negative feedback loop which may be responsible for maintaining the meiotic period.

Introduction

The meiotic cell cycle of an oocyte during oogenesis requires strict regulation to ensure appropriately timed progression as well as arrest. Cdk1 regulates the M phase in both meiosis and mitosis, and acts as an M phase- (or maturation-) promoting factor (MPF) (see review; Morgan, 1995; Nurse, 1990). Cdk1 activity during the M phase is regulated by cyclin B. Cdk1 binds cyclin B, and its activity is maintained at a high level during metaphase, with proteolytic destruction of cyclin B resulting in the loss of Cdk1 activity and exit from metaphase (Glotzer et al., 1991; King et al., 1995). Proteolysis of cyclin B is dependent on the N-terminal region, which is termed the destruction box, and is required for the polyubiquitination mediated by the anaphase promoting complex/cyclosome (APC/C). The truncation mutant of cyclin B, lacking the N-terminal 85–90 amino acids (ΔN-cyclin B), stably activates MPF without requiring APC/C involvement (Iwabuchi et al., 2000; Murray et al., 1989).

The cytoplasmic agents responsible for cell cycle arrest at the metaphase of meiosis II (meta-II) in a mature frog egg are termed cytostatic factors (CSF) (Masui and Markert, 1971), and Mos, the product of the proto-oncogene *c-mos*, plays a main role in CSF activity (Sagata et al., 1989). Mos is a member of the MAP kinase

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cascade, and functions as a MAPK kinase (MEK kinase), activating the MAPK kinase MEK, and resulting in the phosphorylation and activation of the MAPK ERK (see review; Tunquist and Maller, 2003). Thus, the Mos/MEK/ERK pathway is a CSF, and controls meiosis in the eggs of many species (Masui, 2000).

$[Ca^{2+}]_i$ transients are observed in the fertilized eggs of all species investigated (Miyazaki and Ito, 2006; Stricker, 1999), and seem to be critical for initiating several events related to egg activation and cell cycle control (Miyazaki and Ito, 2006; Runft et al., 2002). Initially, the $[Ca^{2+}]_i$ transients in the egg are induced by sperm–egg binding or fusion. In many species, including mammals, a soluble cytoplasmic factor in the sperm (the sperm factor) initiates the $[Ca^{2+}]_i$ transients when the factor intrudes into the egg cytoplasm after sperm–egg fusion (Kyozuka et al., 1998; Oda et al., 1999; Stricker, 1999; Swann, 1990; Yamamoto et al., 2001). Phospholipase Cζ (Saunders et al., 2002) and citrate synthase (Harada et al., 2007) have been identified as the sperm factors from mammals and newts, respectively. Alternatively, the $[Ca^{2+}]_i$ transients of the amphibian Xenopus laevis egg was induced by the RGD peptides and the sperm surface disintegrin peptide (Iwao and Fujimura, 1996; Shilling et al., 1998), which may act on the egg surface protein (Sato et al., 2006). The progression of $[Ca^{2+}]_i$ transients in the egg is generally a taxonomic group-specific feature (Stricker, 1999), and appear to be related to the stage of cell cycle where growth is arrested (Levasseur and McDougall, 2000).

$[Ca^{2+}]_i$ transients mediate Cdk1 and MAPK activities, and downregulate ERK-like kinase activity that seems to drive G1/S transition in sea urchin eggs (Carroll et al., 2000; Kumano et al., 2001; Philipova et al., 2005). In mouse eggs, $[Ca^{2+}]_i$ oscillations decreases cyclin B and Cdk1 activity, resulting in release from metaphase arrest (Nixon et al., 2002). Interestingly, Cdk1 and MAPK have been shown to regulate $[Ca^{2+}]_i$ oscillations (Marino et al., 2000; McDougall and Levasseur, 1998; Russo et al., 1996). $[Ca^{2+}]_i$ transients in the eggs of most animals are mediated by the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) (Deguchi and Morisawa, 2003; Stricker, 1999), and activation of either MPF or MAPK sensitizes IP_3-dependent Ca^{2+} release, possibly through direct phosphorylation of the IP_3R (Sun et al., 2009). Thus, the Cdk1 and ERK activities and $[Ca^{2+}]_i$ oscillations in the egg regulate each other. However, the mechanisms by which ERK and Cdk1 regulate $[Ca^{2+}]_i$ oscillations are still controversial. In the egg of the ascidian Ascidiella aspersa, MAPK activity seems to not bear a temporal correlation with $[Ca^{2+}]_i$ oscillations (McDougall and Levasseur, 1998), and the inactivation of MAPK has no effect on $[Ca^{2+}]_i$ oscillations and meiotic division at fertilization (Levasseur and McDougall, 2000; McDougall and Levasseur, 1998). Alternatively, the Mos/MEK/ERK pathway seems to regulate $[Ca^{2+}]_i$ oscillations in the mouse (Lee et al., 2006) and maintain meiotic division in ascidians (Dumollard et al., 2011). Thus, the exact role of the Mos/MEK/ERK pathway in $[Ca^{2+}]_i$ oscillations and the cell cycle is still unknown.

In ascidians, mature eggs are arrested at the meta I stage (Dale, 1983), and 2 distinct sequences of $[Ca^{2+}]_i$ oscillations are also observed after fertilization: phase I $[Ca^{2+}]_i$ oscillations appear before the first polar body extrusion, and phase II $[Ca^{2+}]_i$ oscillations appear between the extrusion of the first and second polar bodies (Brownlee and Dale, 1990; McDougall and Levasseur, 1998; Russo et al., 1996; Sensui and Morisawa, 1996; Speksnijder et al., 1989; Yoshida et al., 1998). The $[Ca^{2+}]_i$ transients in the egg are crucial for the extrusion of the polar bodies (McDougall and Sardet, 1995; Sensui and Morisawa, 1996; Yoshida et al., 1998), and therefore, the ascidian egg is a good model for studying the relationship between meiosis and Ca^{2+}. In the present study, we investigated the role of Mos/MEK/ERK pathway in $[Ca^{2+}]_i$ oscillations observed in the eggs of the ascidian Phallusia nigra. We showed that the Mos/MEK/ERK pathway is essential for $[Ca^{2+}]_i$ oscillations, in particular, the phase II $[Ca^{2+}]_i$ oscillations in the ascidian egg.

Materials and methods

Materials and solutions

The ascidian P. nigra was collected from the piers or rafts located on the west coast of Okinawa island (Japan). The animals were kept in an aquarium at 15–25 °C. Eggs and sperm were obtained from the oviducts and spermiducts by dissection. The vitelline envelope and accessory cells around the egg cells were removed by immersion in artificial seawater (ASW) containing 1% sodium thioglycolate and 0.05% actinase E (Kaken Pharmaceutical Co., Tokyo, Japan) for 20–60 min.

The ASW consisted of 462 mM NaCl, 9.4 mM KCl, 9 mM $CaCl_2$, 28 mM $MgSO_4$, 22 mM $MgCl_2$, and 10 mM HEPES-NaOH (pH 8.2). To activate the sperm, the semen sample was suspended in high pH ASW (pH 9.5), buffered with 10 mM CHES-NaOH, for 30–120 min before insemination. Subsequently, the pH of the sperm suspension was decreased to pH 8.2 by adding ASW (pH 6.5) buffered with 10 mM MES-NaOH just before the insemination. A glutathione S-transferase (GST) fusion protein containing the starfish Mos protein (GST-Mos) and the control protein (GST alone) were prepared, as described previously (Tachibana et al., 2000). A nondegradable fragment of the Xenopus cyclin B2 GST-fusion protein (GST-ΔN85-cyclin) was prepared, as described previously (Iwabuchi et al., 2000). U0126 (Merck, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (DMSO) at 10 mM for use as the stock solution, and 10 μM solution was prepared in ASW containing 0.5% DMSO just before application to the oocytes.

Microinjection and measurement of intracellular Ca^{2+}

Microinjection of Calcium green-1 dextran (Mr 10000; CaG1dx; Invitrogen, Carlsbad, CA) was performed, as previously described (Sensui and Morisawa, 1996). 250 μM CaGldx was dissolved in the injection buffer (50 mM KCl, 100 μM EGTA 10 mM Hepes-KOH), which was microinjected into the egg, yielding a cytoplasmic concentration of 5–10 μM. The volume was estimated by measuring the diameter of the droplets injected into the silicone oil, and the changes in the meniscus of the injected reagents in the micropipette. GST, GST-Mos, and GST-ΔN85-cyclin were co-injected with CaG1dx. The eggs were exposed to the excitation beam through a 470–490 nm band-pass filter, and fluorescence from the egg was collected for 400 ms at 4-s intervals with a 515–550 nm band-pass filter on an inverted microscope (IX-70; Olympus, Tokyo, Japan). The images were captured with a digital camera (RETIGA Exi, QI imaging), transferred to a computer (MacPro; Apple, Cupertino, CA), and analyzed with a TI Workbench (see; Yoshida et al., 1998).

Preparation of ascidian egg extracts

Twenty-five oocytes, eggs, or embryos were suspended into 30 μL of ASW, and the suspensions were centrifuged using a hand-driven centrifuge for 10 s. After removing the supernatants (27 μL), the settled eggs were immediately frozen in liquid nitrogen. Following thawing, the pellet was mixed with 22 μL of extraction buffer (160 mM Na-β-glycerophosphate, 40 mM EGTA, 30 mM $MgCl_2$, 200 mM KCl, 200 mM sucrose, 2 mM DTT, 1 mM Na-orthovanadate, 0.1% Nonidet P-40, 0.5 mM benzamidine,

0.5 mM PMSF, 50 µg/mL leupeptin, and 50 µg/mL soybean trypsin inhibitor, pH 7.3), and homogenized by vortexing for 3 s. After centrifugation at 12,000g for 10 min at 4 °C, the supernatant was recovered to a fresh tube. For immunoblots, 10 µL of the extract was added to 10 µL of 2x concentrated sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer, followed by boiling for 5 min.

Histone H1 kinase assay

The kinase assays contained 4 µL of the oocyte, egg, or embryo suspension, and 4 µL of the histone H1-ATP mixture with a final concentration of 0.3 mg/mL of histone H1 (Roche, Basel, Switzerland), 10 µM of cold ATP, and 5.92 MBq/mL of $[\gamma\text{-}^{32}P]$ATP. The kinase assay mixture was incubated for 30 min at 25 °C, and the reaction was terminated by the addition of 4 µL of 3x concentrated SDS-PAGE sample buffer followed by boiling for 5 min. Samples were analyzed by 12.5% SDS-PAGE, and the gel was autoradiographed with an x-ray film (RX-U; Fuji Film, Tokyo, Japan). The radioactivity of the excised histone H1 bands was quantified using a liquid scintillation counter.

Immunoblotting analysis of ERK

For immunoblotting, samples (each of which contained a lysate of 30 eggs (non-injected oocyte) or 6 eggs (injected) depending upon experiments) were separated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioTrace NT, Pall). Membranes were blocked for 30 min with TBS supplemented with 0.05% Tween-20 (TBST) and 5% skim milk (BD, Franklin Lakes, NJ), and incubated overnight at 4 °C with the primary antibody (1:1000): rabbit polyclonal anti-ERK1/2, CT antibody (Upstate Biotechnology, Lake Placid, NY) or rabbit polyclonal anti-phosphorylated ERK1/2 antibody (Sigma, St. Louis, MO). After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Buckinghamshire, England) at a dilution of 1:10,000 for 1 h at room temperature. After washing with TBST, the membrane was incubated with enhanced chemiluminescence (ECL) advance western blotting detection reagents (Amersham) for 5 min at room temperature, followed by exposure to luminescent image analysis (LAS1000 Plus, Fuji) to obtain the luminescent image. To reprobe with another antibody, immunoblotted membranes were stripped with WB Stripping Solution Strong (Nacalai Tesque, Kyoto, Japan).

Results

Changes in $[Ca^{2+}]_i$ and ERK and H1K activities in the egg during fertilization and cleavage

First, we analyzed $[Ca^{2+}]_i$ and ERK and Cdk1 activities in the egg of the ascidian *P. nigra*. The *Phallusia* egg showed two series of post-fertilization $[Ca^{2+}]_i$ oscillations. The first series of $[Ca^{2+}]_i$ oscillations (phase I $[Ca^{2+}]_i$ oscillations) occurred soon after insemination and lasted for about 5 min until extrusion of the first polar body (Fig. 1A). The fertilized ascidian egg shows cortical contractions with the egg deformations mediated by the cortical network of actin filaments (Sawada and Osanai, 1985), which is also mediated by $[Ca^{2+}]_i$ oscillations (Sensui and Morisawa, 1996; Yoshida et al., 2003; Yoshida et al., 1998). Similar to other ascidian species, egg deformation was also observed during phase I $[Ca^{2+}]_i$ oscillation in the *P. mammillata* egg. Egg deformation is the first morphological change after fertilization; therefore, we
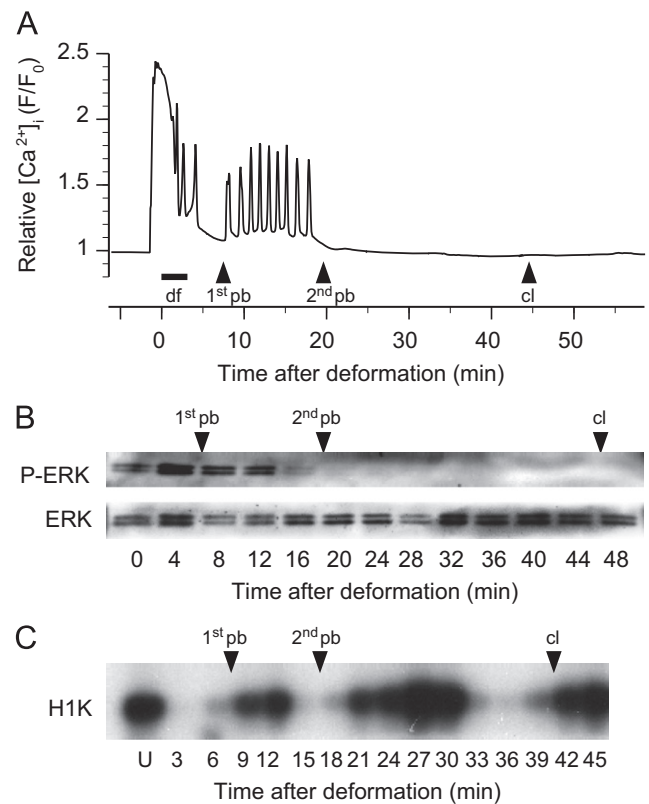

Fig. 1. Changes in intracellular Ca^{2+} concentration, ERK phosphorylation, and H1K activity following fertilization in the *Phallusia nigra* egg. (A) Changes in $[Ca^{2+}]_i$ of the egg after fertilization. The Ca^{2+} sensitive dye Calcium green-1 dextran ($Mr = 10,000$) was used for measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was evaluated by the ratio of fluorescence intensity at a time point (F) to that at the start of the measurement (F_0). Since it is difficult to know the exact time of fertilization, we used the first signs of egg deformation as the reference time. $[Ca^{2+}]_i$ oscillations ceased after meiosis. (B) Dynamics of ERK after fertilization. Phosphorylated-ERK (P-ERK) and ERK were immunoblotted with their respective antibodies. Arrowheads indicate the timing of the extrusion of the first polar body (1st pb), the extrusion of the second polar body (2nd pb), and the first cleavage (cl). We regarded the 2 bands at 44 and 42 kDa recognized by the 2 antibodies as ERK1/2. Phosphorylated ERK proteins were present during meiosis, and then disappeared after meiosis. (C) Dynamics of Cdk1 activity in the egg after fertilization. The activity was evaluated by the phosphorylation of histone H1 with the help of an autoradiogram (see Materials and Methods). Arrowheads indicate the timing of the extrusion of the first polar body (1st pb), the extrusion of the second polar body (2nd pb), and the first cleavage (cl).

used this phenomenon as the fiducial mark for fertilization. The second series of $[Ca^{2+}]_i$ oscillations (phase II $[Ca^{2+}]_i$ oscillations) was observed in the egg at 0.5–2 min after the end of phase I in which the first polar body was extruded, and it ceased after the extrusion of the second polar body. Thereafter, no $[Ca^{2+}]_i$ transients were observed after meiosis (Fig. 1A). These $[Ca^{2+}]_i$ oscillations patterns are similar to those observed in other ascidians (McDougall and Levasseur, 1998; Russo et al., 1996; Yoshida et al., 1998).

Immunoblotting using an anti-pan ERK antibody identified 39 kDa and 41 kDa proteins in the unfertilized egg of *P. nigra* (Fig. 1B); we concluded that these are the ERK1/2 of *P. nigra*. There is no difference in the signals between the two ERK bands in the all immunoblotting we have performed. The ERK were roughly preserved from the pre-fertilization stage until the cleavage stage (Fig. 1B). However, the signal intensities of the active ERKs detected by an antibody against phosphorylated ERK gradually decreased after the extrusion of the first polar body and eventually disappeared at the extrusion of the second polar body, and it was still absent during the cleavage stage of 1 cell to 2 cells (Fig. 1B).

Next, we examined Cdk1 activity in the *Phallusia* egg by assaying Histone H1 kinase activity. Unfertilized eggs are arrested at meiotic metaphase I (Meta-I), and the histone H1 kinase activity, demonstrating Cdk1 activity, in the egg was high (Fig. 1C). The kinase activity decreased to undetectable levels immediately after fertilization, and then increased periodically according to the meiotic and mitotic cell cycles (Fig. 1C).

ERK inactivation diminishes [Ca²⁺]ᵢ oscillations during meiosis II

The effect of ERK on $[Ca^{2+}]_i$ oscillations was examined using U0126, an inhibitor of MEK. MEK is an ERK kinase, which phosphorylates ERK resulting in its activation. When the unfertilized eggs were incubated with U0126 (10 μM) for 5–10 min before insemination, phosphorylated ERK (ERK active form) was not observed after fertilization (Fig. 2A); notably, ERK activity is maintained during normal meiosis (Fig. 1B). Amounts of ERK did

not change during meiosis and after the mitotic stage, even in the presence of 10 μM U0126 (Fig. 2A). Interestingly, when treated with U0126, the first polar body was extruded normally, but cytokinesis during the extrusion of the second polar body and the first cleavage was incomplete. In these cases, cleavage furrows appeared, but did not contract completely or sever the polar body and blastomeres.

U0126 also disrupted $[Ca^{2+}]_i$ oscillations. In the presence of U0126 (10 μM), phase I $[Ca^{2+}]_i$ oscillations were still observed in all cases, but phase II $[Ca^{2+}]_i$ oscillations disappeared completely in 17 of 24 cases (Fig. 2B), or were greatly reduced to 1–3 small $[Ca^{2+}]_i$ transients in 7 cases (Fig. 2C). DMSO (0.5%), which was used to solubilize U0126, had no effect on either phases of $[Ca^{2+}]_i$ oscillations (n=14); data not shown. Another MEK inhibitor, CI-1040 also showed similar results to U0126 (Fig. S1). These results suggest ERK activity is necessary for maintenance of $[Ca^{2+}]_i$ oscillations during second meiosis and cytokinesis.

Mos maintains ERK activity and [Ca²⁺]ᵢ oscillations

Active ERK seems to be necessary for $[Ca^{2+}]_i$ oscillations during second meiosis. Therefore, we next examined the effect of ERK activation on $[Ca^{2+}]_i$ oscillations by using the Mos protein, an activator of MEK, which induces ERK phosphorylation and activation. When 144 pL of 0.42 mg/mL GST-conjugated Mos protein (GST-Mos) was injected into the unfertilized eggs (final concentration, 44 μg/mL in the egg), the phosphorylation of ERK was preserved for at least 120 min after fertilization (Fig. 3A). Alternatively, phosphorylation disappeared after the extrusion of second polar body (within 20 min) when the GST control protein was injected instead of GST-Mos (Fig. 3B). When GST-Mos was injected into the egg just after the extrusion of the second polar body, ERK was phosphorylated again within 10 min after the injection, and the activated ERK was retained for at least 125 min (Fig. 3C). ERK levels did not change even in the presence of GST-Mos or GST (Fig. 3A–C).

In the eggs injected with GST-Mos, the phase II $[Ca^{2+}]_i$ oscillations lasted for 19.8 ± 8.0 min after the second polar body extrusion (n=12; Fig. 3D). Sometimes, a 2–7 min gap appeared between the prolonged $[Ca^{2+}]_i$ oscillations during second polar body extrusion; after $[Ca^{2+}]_i$ oscillations, 1–3 $[Ca^{2+}]_i$ transients periodically appeared with intervals of 13–20 min (5 of 12 cases; Fig. 3D). When GST-Mos was injected into the eggs just after the extrusion of the second polar body, $[Ca^{2+}]_i$ transients occurred 10–20 min after the injection and lasted at least 120 min (n=11; Fig. 3E).

The GST-Mos-injected eggs showed extrusions of first and second polar bodies, but not cleavage at least for 129 min after fertilization. Periodically, the GST-Mos-injected eggs showed cortical contractions (gray arrowheads in Fig. 3D, E) after the $[Ca^{2+}]_i$ transients. Furthermore, cytokinesis-like deformations appeared after the $[Ca^{2+}]_i$ transients in the GST-Mos-injected eggs following the extrusion of the second polar body. Alternatively, the eggs injected with only GST did not show $[Ca^{2+}]_i$ oscillations after the extrusion of the second polar body, and cleavage occurred normally (n=11; Fig. 3F, G).

Cyclin B maintained, but did not induce ERK phosphorylation and [Ca²⁺]ᵢ oscillations

Our results showed that ERK is responsible for $[Ca^{2+}]_i$ oscillations even after the extrusion of the second polar body, i.e., in the post-meiotic period. In addition, ΔN-cyclin B, which is not destroyed by the proteasome and stably activates Cdk1, prolongs the duration of $[Ca^{2+}]_i$ oscillations in another ascidian, *Ascidiella aspersa* (Levasseur and McDougall, 2000) (see Fig. S2). Therefore,
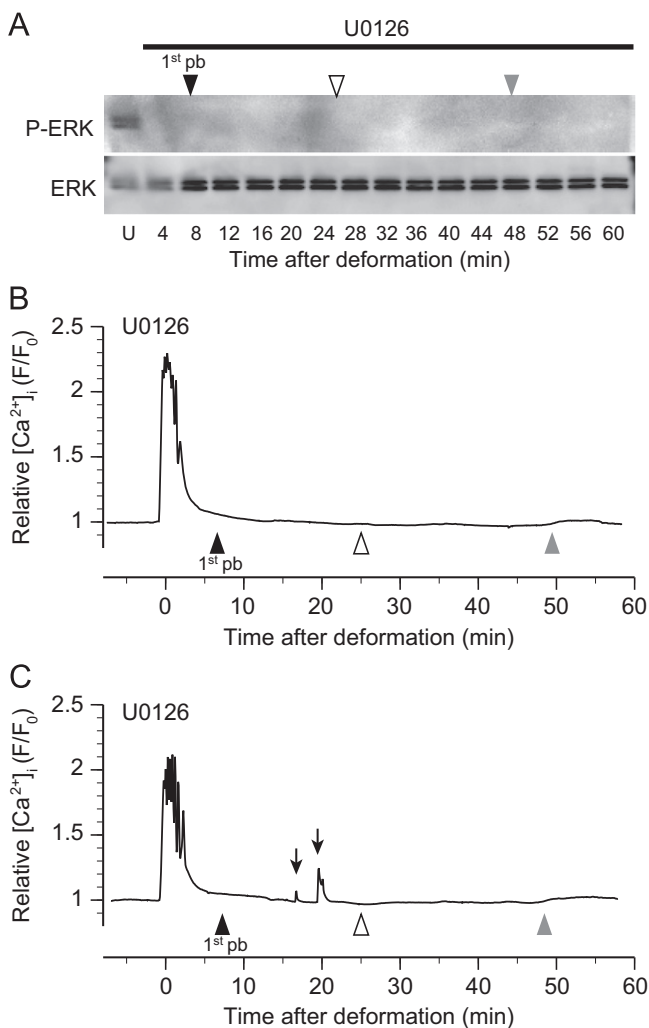
Fig. 2. Effect of the MEK inhibitor U0126 on the egg. (A) Dynamics of ERK in the egg after fertilization and treatment with 10 μM U0126. In the experiment, U0126 was applied to the unfertilized egg at 5–10 min before insemination. ERK activity existed in the unfertilized, untreated egg (U), but the activity diminished immediately after treatment with U0126. (B, C) Changes in [Ca²⁺]ᵢ of the egg under the influence of 10 μM U0126 after fertilization. Arrowheads indicate the timing of extrusion of the first polar body (black), second polar body-like protrusion (white), and first cleavage-like furrow formation (gray). (B) Example of the samples without phase II [Ca²⁺]ᵢ oscillations (17 of the 24 analyzed). (C) Samples showing several small [Ca²⁺]ᵢ transients (arrows) 20–30 min after the first [Ca²⁺]ᵢ transient (7/24). U0126 itself has no effect on the IP₃R sensitivity in the egg (see Fig. S3).
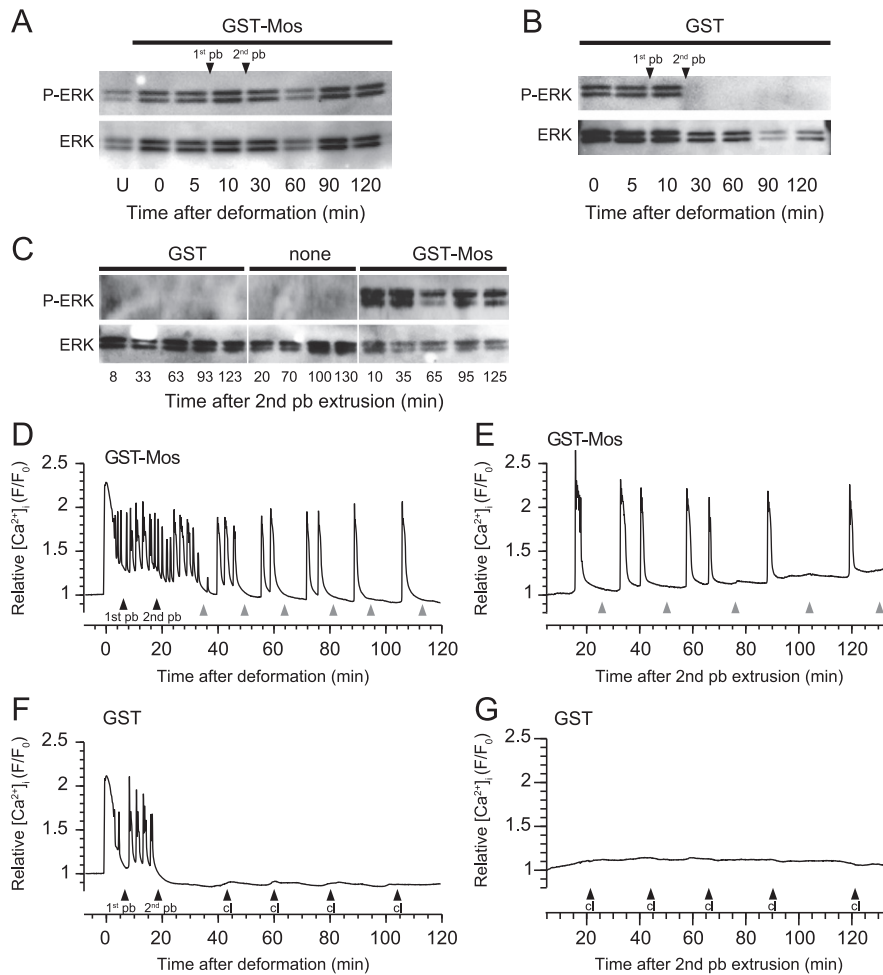
Fig. 3. GST-Mos maintained $[Ca^{2+}]_i$ oscillations after the extrusion of the second polar body. (A, B) Dynamics of ERK after fertilization in the egg injected with GST-Mos (A) and GST (B) before insemination. GST-Mos maintained ERK activity. Arrowhead indicates the timing of polar body extrusions. (C) Dynamics of ERK in the egg after the extrusion of second polar body. The eggs were injected with GST-Mos and GST after the second polar body extrusion, and some non-injected eggs were used as controls. GST-Mos induced elevated ERK activity, even when GST-Mos was injected into eggs after the second polar body extrusion. (D–G) $[Ca^{2+}]_i$ oscillations in the egg injected with GST-Mos (D, E) or GST (F, G). Eggs injected with GST-Mos before insemination showed $[Ca^{2+}]_i$ oscillations after the second polar body extrusion (D). When GST-Mos was injected eggs after the second polar body extrusion, $[Ca^{2+}]_i$ oscillations were induced, and they lasted for > 120 min (E). Eggs that were injected with GST before insemination (F) or after the second polar body extrusion (G) showed no $[Ca^{2+}]_i$ oscillations after the extrusion of the second polar body, as observed under normal conditions (see Fig. 1A). Black arrowheads indicate the timing of polar body extrusions (1st and 2nd pb), and cleavages (cl). Gray arrowheads show the timing of cytokinesis-like contractions of the egg.

we examined the effect of Cdk1 and ERK on $[Ca^{2+}]_i$ oscillations. When GST-ΔN85-cyclin (ΔN-cyclin B) was injected into the *Phallusia* eggs before insemination (6.4 μg/mL in the egg), phosphorylated ERK was present for over 120 min after fertilization (Fig. 4A left). Moreover, $[Ca^{2+}]_i$ oscillations were prolonged and the extrusions of the polar bodies were blocked ($n=11$; Fig. 4C). Alternatively, when GST-ΔN85-cyclin was injected into the oocyte just after the extrusion of the second polar body, $[Ca^{2+}]_i$ oscillations were not observed; GST-ΔN85-cyclin had no effects on $[Ca^{2+}]_i$ after the extrusion of the second polar body, even at a high concentration (27 μg/ml; $n=8$; Fig. 4D). In this case, phosphorylation of ERK after injection was not observed (Fig. 4A, right). These results suggest that Cdk1 can maintain, but not activate ERK and $[Ca^{2+}]_i$ oscillations.

U0126 terminates Cdk1-mediated $[Ca^{2+}]_i$ oscillations and ERK phosphorylation

As described above, GST-ΔN85-cyclin prolonged, but did not activate ERK and $[Ca^{2+}]_i$ oscillations. Thus, we examined the relationship between ERK, Cdk1, and $[Ca^{2+}]_i$ oscillations by using U0126. When 10 μM of U0126 was applied to the

GST-ΔN85-cyclin-injected egg 30 min after fertilization, the active ERK disappeared within 3 min (Fig. 4B), even though the Cdk1 activity kept high (Fig. S2). This activity level was similar to that observed in the normal fertilized oocyte (Fig. 2A). $[Ca^{2+}]_i$ oscillations in the eggs also disappeared within 10 min of the application (7 out of 12; Fig. 4E) or was reduced to 1–2 small $[Ca^{2+}]_i$ transients that appeared at about 50 min after fertilization (5 out of the 12; Fig. 4G). Thus, Cdk1-mediated $[Ca^{2+}]_i$ oscillations seem to require ERK activity.

Cyclin B increased the frequency of $[Ca^{2+}]_i$ transients in the Mos-induced $[Ca^{2+}]_i$ oscillations after the second polar body extrusion

Our results show that Mos can trigger $[Ca^{2+}]_i$ oscillations after the second polar body extrusion, whereas GST-ΔN85-cyclin cannot, although it prolongs the duration of $[Ca^{2+}]_i$ oscillations. Therefore, we examined the role of Cdk1 on Mos-induced $[Ca^{2+}]_i$ oscillations after the second polar body extrusion. The eggs in which GST-Mos was injected after the second polar body extrusion showed $[Ca^{2+}]_i$ oscillations (Fig. 4F). The average number of $[Ca^{2+}]_i$ transients during the first 60 min following the first transient after the second polar body extrusion was 3.6 ± 0.6
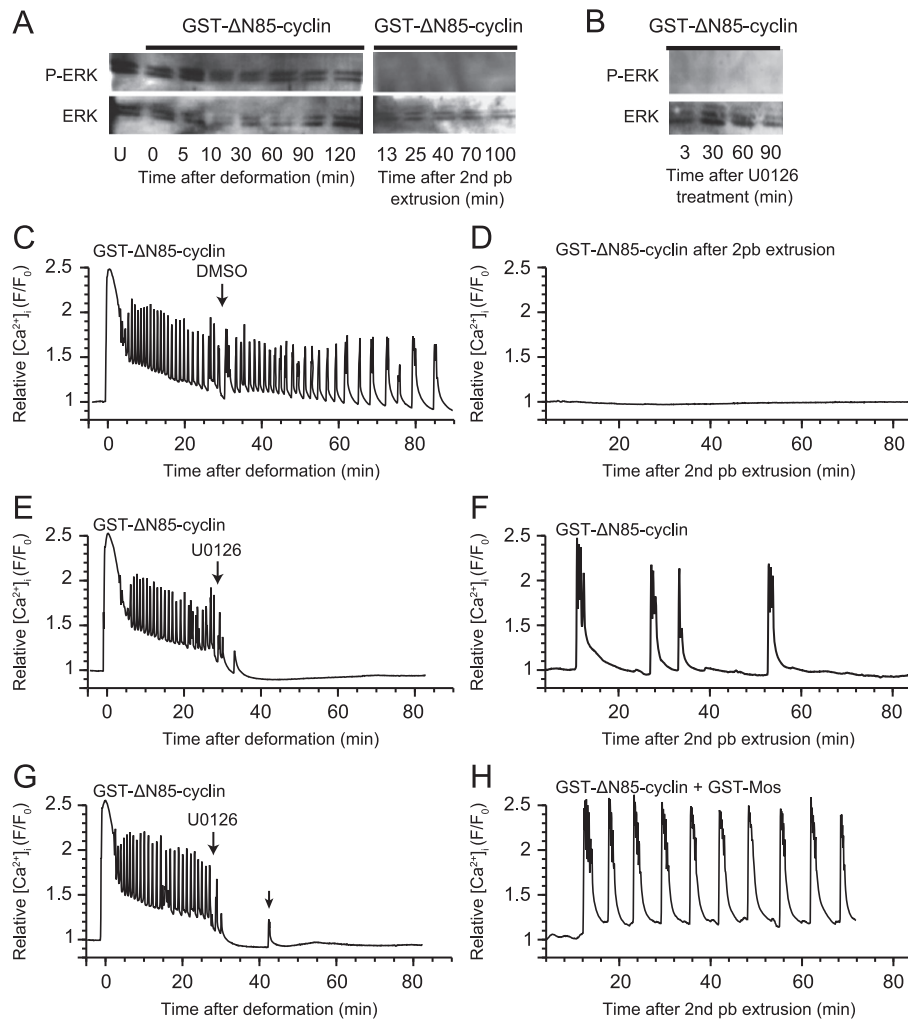
Fig. 4. U0126 diminished the ERK activity and $[Ca^{2+}]_i$ oscillations sustained by GST-ΔN85-cyclin. (A) (Left) Post-fertilization dynamics of ERK in the egg injected with GST-ΔN85-cyclin prior to insemination. U indicates the sample of unfertilized eggs. (Right) Post-fertilization dynamics of ERK in the egg injected with GST-ΔN85-cyclin after the extrusion of the second polar body. The ERK activity was sustained for over 2 h in the egg injected with GST-ΔN85-cyclin prior to insemination. Alternatively, ERK activity in the egg was decreased when GST-ΔN85-cyclin was injected after the extrusion of the second polar body. (B) Post-fertilization dynamics of ERK in the GST-ΔN85-cyclin-injected eggs under the influence of U0126. U0126 treatment started 30 min after fertilization, and immediately diminished the ERK activity in the egg. (C, E, G) Post-insemination changes in $[Ca^{2+}]_i$ in the egg injected with GST-ΔN85-cyclin before insemination. DMSO (C) or U0126 (E, G) was added to the medium at the times indicated (arrows). U0126 terminated $[Ca^{2+}]_i$ oscillations in the GST-ΔN85-cyclin-injected egg. (D, F, H) Changes in $[Ca^{2+}]_i$ of the egg after the second polar body extrusion. Observation of $[Ca^{2+}]_i$ was started at about 4 min after the second polar body extrusion. (D) GST-ΔN85-cyclin was injected into the egg just after the extrusion of the second polar body. $[Ca^{2+}]_i$ oscillations in the egg, and cleavages were not observed. Post-meiosis injection of GST-ΔN85-cyclin induced neither ERK activation nor $[Ca^{2+}]_i$ transient. (F) GST-ΔN85-cyclin only or (H) GST-Mos and GST-ΔN85-cyclin were injected into the egg immediately after the extrusion of the second polar body. Injection of GST-ΔN85-cyclin enhanced the frequency of $[Ca^{2+}]_i$ oscillations in the GST-Mos-injected egg. U0126 itself has no effect on the IP$_3$R sensitivity in the egg (see Fig. S3).

($n=16$). Surprisingly, GST-ΔN85-cyclin increased the frequency of Mos-induced $[Ca^{2+}]_i$ oscillations. When GST-ΔN85-cyclin (1.38 μg/mL in the egg) was injected into the oocytes in addition to GST-Mos (28 μg/mL in the egg), the number of the $[Ca^{2+}]_i$ transients increased to 9.6 ± 2.8 ($n=20$; Fig. 4F, H). Therefore, the frequency of $[Ca^{2+}]_i$ oscillations increased 2.7-fold compared to those following the injection of GST-Mos alone (Fig. 4H).

Discussion

Mos/MEK/ERK pathway and $[Ca^{2+}]_i$ oscillations

In ascidian eggs, Cdk1 activation reciprocally interacts with $[Ca^{2+}]_i$ oscillations after fertilization; as active Cdk1 induces $[Ca^{2+}]_i$ oscillations, whereas high $[Ca^{2+}]_i$ induces degradation of cyclin B resulting in decreased Cdk1 activity (Levasseur and McDougall, 2000). However, regulation of $[Ca^{2+}]_i$ transients by

the Mos/MEK/ERK pathway has yet to be observed in detail. In this study, we showed that phase II $[Ca^{2+}]_i$ oscillations are diminished by inhibition of the Mos/MEK/ERK pathway, but the phase I $[Ca^{2+}]_i$ oscillations are not affected by the pathway. Furthermore, GST-Mos prolonged the duration of $[Ca^{2+}]_i$ oscillations in the post-meiotic period. Thus, the activity of the Mos/MEK/ERK pathway is indispensable and sufficient for phase II $[Ca^{2+}]_i$ oscillations in the *Phallusia* egg. In another ascidian, namely, *Ascidiella aspersa*, MAPK activity does not seem to be a temporal correlation with $[Ca^{2+}]_i$ oscillations, since the MAPK kinase inhibitor U0126 has no effect on $[Ca^{2+}]_i$ oscillations and meiotic division at fertilization (Levasseur and McDougall, 2000; McDougall and Levasseur, 1998). The discrepancy in the results may be derived from the differences in the permeability and/or sensitivity to U0126 among the eggs of these ascidian species.

Increased $[Ca^{2+}]_i$ seems to inactivate MAPK activity in the sea urchin egg (Zhang et al., 2006), and in our preliminary observations, Ca^{2+} decreased ERK activity in the artificially MII-arrested

oocytes of the ascidian *Ciona savignyi* (unpublished data). Thus, ERK activity and $[Ca^{2+}]_i$ oscillations probably form a negative feedback loop, with active ERK maintaining $[Ca^{2+}]_i$ oscillations, and $[Ca^{2+}]_i$ oscillations inactivating ERK, resulting in the termination of $[Ca^{2+}]_i$ oscillations.

$[Ca^{2+}]_i$ oscillations after the second polar body extrusion

ERK plays an important role in meiosis and $[Ca^{2+}]_i$ oscillations in the eggs. $[Ca^{2+}]_i$ oscillations are not usually observed after the extrusion of the second polar body; however, artificially activated ERK altered this, inducing $[Ca^{2+}]_i$ oscillations after the extrusion of the second polar body (Fig. 3). Indeed, $[Ca^{2+}]_i$ oscillations persisted during conditions of high ERK activity, and were terminated when ERK was inactivated, even in the post-meiotic period. These results suggest that the machinery involved in $[Ca^{2+}]_i$ oscillations that is regulated by the Mos/MEK/ERK pathway is preserved in the zygote, even after the end of meiosis. Cessation of $[Ca^{2+}]_i$ oscillations seems to be concomitant with ERK inactivation.

The Mos/MEK/ERK pathway acts as a CSF in many species (Hashimoto et al., 1994; Kondoh et al., 2006; Mori et al., 2006; Sagata et al., 1989; Tachibana et al., 2000; Yamamoto et al., 2008), and maintains the important process of meiotic arrest. Recently, the maintaining activity of the Mos/MAPK pathway after fertilization was shown to induce additional rounds of meiotic M phase in other ascidian eggs (Dumollard et al., 2011). Thus, the Mos/MEK/ERK pathway seems to maintain meiotic division without entering mitotic division, and the loss of Mos/MAPK activity seems to be necessary for preventing entry into further rounds of meiosis (Dumollard et al., 2011). In the present study, increased levels of active ERK induced by GST-Mos also blocked cleavage of the ascidian egg, but did not interfere with the extrusion of polar bodies. We also observed that the GST-Mos-injected eggs periodically showed cortical contractions (Fig. 3). Thus, it is plausible that the Mos/MEK/ERK pathway maintains meiotic division without entering mitotic division in our experiments; implying that $[Ca^{2+}]_i$ oscillations observed occurred during the meiotic period. Interestingly, activation of the Mos/MEK/ERK pathway after meiosis also induced $[Ca^{2+}]_i$ oscillations with periodic cortical contractions (Figs. 3E, 4F). In these cases, it is still unknown whether the Mos/MEK/ERK pathway induces re-entry into the meiotic stage, or induces $[Ca^{2+}]_i$ oscillations in the mitotic stage.

If the Mos/MEK/ERK pathway maintains the meiotic stage, it is important to understand the relationship between Ca^{2+} and meiosis. As described above, the $[Ca^{2+}]_i$ transients mediate Cdk1 and MAPK activities. Previously, we showed that $[Ca^{2+}]_i$ transients are sufficient to drive metaphase-anaphase transition in the meiosis of the ascidian *C. savignyi* (Sensui and Morisawa, 1996; Yoshida et al., 1998). Furthermore, post-activation Ca^{2+} waves may be required for the extrusion of the second polar body in the ascidian *P. mammillata* (McDougall and Sardet, 1995). Thus, it is probable that the termination of $[Ca^{2+}]_i$ oscillations may induce the end of the meiotic period. As described above, ERK activity and $[Ca^{2+}]_i$ oscillations seem to form a negative feedback loop; thus, the ERK/Ca^{2+} oscillations feedback loop maintains the meiotic period.

Effect of Cdk1 on $[Ca^{2+}]_i$ oscillations

Cyclin B1 is a regulatory component of Cdk1, and plays a major role in the promotion and preservation of metaphase. In another ascidian, *A. aspersa*, ΔN-cyclin B steadily activates Cdk1 and prolongs the sperm-induced $[Ca^{2+}]_i$ oscillations (Levasseur and McDougall, 2000). Furthermore, it has been shown that Cdk1 activity is both necessary and sufficient for phase II $[Ca^{2+}]_i$

oscillations (Levasseur et al., 2007). In the present study, GST-Δ85N-cyclin B also maintained ERK activity and prolonged the period of $[Ca^{2+}]_i$ oscillations in the *Phallusia* eggs; the period of $[Ca^{2+}]_i$ oscillations correlated with both ERK and Cdk1 activities. Furthermore, GST-ΔN85-cyclin in the presence of GST-Mos enhanced the frequency of $[Ca^{2+}]_i$ oscillations after the second polar body extrusion (Fig. 4). Thus, ERK and Cdk1 synergistically induce $[Ca^{2+}]_i$ oscillations.

Alternatively, U0126 terminated the prolonged $[Ca^{2+}]_i$ oscillations induced by GST-Δ85N-cyclin B. Furthermore, GST-Δ85N-cyclin B could not elevate ERK activity as well as induce $[Ca^{2+}]_i$ oscillations after the second polar body extrusion. These results show that Cdk1 can only mediate $[Ca^{2+}]_i$ oscillations in the presence of activated ERK. It is possible that Cdk1 maintains the ERK activity and $[Ca^{2+}]_i$ oscillations, but Cdk1 is not essential for $[Ca^{2+}]_i$ oscillations. Sustained activity of the Mos/MAPK pathway seems to keep the ascidian eggs in the meiotic stage (Dumollard et al., 2011), and activation of the Mos/MEK/ERK pathway induces $[Ca^{2+}]_i$ oscillations with periodic cortical contractions even in the mitotic period (Figs. 3E, 4F). Actually, the Cdk1 activity in the GST-Mos-injected egg also changes periodically (Fig. S2). Thus, it is possible that the Mos/MEK/ERK pathway induces the meiotic period, and Cdk1 induces $[Ca^{2+}]_i$ oscillations during this period. In addition, decreased Cdk1 activity may lead to the inactivation of ERK and the end of meiosis, resulting in the termination of $[Ca^{2+}]_i$ oscillations.

Mos/MEK/ERK pathway and IP₃R-induced $[Ca^{2+}]_i$ oscillations

In the ascidian egg, phase II $[Ca^{2+}]_i$ oscillations are driven by the IP₃R that is responsible for Ca^{2+} release from an internal store (Russo et al., 1996; Yoshida et al., 1998). In mouse and *Xenopus* eggs, IP₃R activity is regulated by its phosphorylation, which is mediated by various kinases, including ERK and Cdk1 (Lee et al., 2006). Furthermore, IP₃R sensitivity is regulated by ERK during the maturation of the *Xenopus* oocytes (Sun et al., 2009). Thus, it is probable that IP₃R activity during phase II $[Ca^{2+}]_i$ oscillations in ascidian eggs is regulated by the Mos/MEK/ERK pathway and that IP₃R activation via the Mos/MEK/ERK pathway induces phase II $[Ca^{2+}]_i$ oscillations, whereas IP₃R inactivation results in the termination of phase II $[Ca^{2+}]_i$ oscillations. However, in the ascidian eggs, IP₃R responsiveness decreases after the extrusion of the second polar body, when Ca^{2+} oscillations have finished (Levasseur and McDougall, 2003). Thus, the Mos/MEK/ERK pathway may regulate not only IP₃R responsiveness but also IP3 concentrations.

Interestingly, phase I $[Ca^{2+}]_i$ oscillations were not affected by U0126, although ERK was inactivated. Indeed, phase I $[Ca^{2+}]_i$ oscillations seems to differ from those of phase II, since phase I could not be inhibited by any IP₃R-related inhibitors, while phase II $[Ca^{2+}]_i$ oscillations seems to be driven by the IP₃R (Yoshida et al., 1998). Furthermore, responsiveness of the IP₃R is maintained throughout meiosis, including phase I and phase II $[Ca^{2+}]_i$ oscillations (Levasseur and McDougall, 2003). Thus, phase I $[Ca^{2+}]_i$ oscillations may not be mediated by the Mos/MEK/ERK pathway, but increases in IP3 induced by factors such as PLC-ζ or citrate synthase may trigger phase I $[Ca^{2+}]_i$ oscillations.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.05.011.

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