

# Start of the embryonic cell cycle is dually locked in unfertilized starfish eggs

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A key event in the oocyte-to-embryo transition is the start of the embryonic mitotic cell cycle. Prior to this start, the cell cycle in oocytes is generally arrested at a particular stage during meiosis, and the meiotic arrest is released by fertilization. However, it remains unclear how release from the meiotic arrest is implicated in the start of the embryonic cell cycle. To elucidate this link, we have used starfish eggs, in which G1 phase arrest occurs after completion of meiosis if the mature oocytes are not fertilized, and fertilization simply directs the start of the embryonic cell cycle. The starfish G1 arrest is known to rely on the Mos-MAPK-Rsk (p90 ribosomal S6 kinase) pathway, and inactivation of Rsk induces S phase in the absence of fertilization. However, here we show that this S phase is not followed by M phase when MAPK remains active, owing to poly(A)-independent repression of cyclin A and B synthesis. By contrast, inactivation of MAPK alone induces M phase, even when S phase is inhibited by constitutively active Rsk. Thus, there is a divergence of separate pathways downstream of MAPK that together block the start of the embryonic mitotic cycle. One is the previously known Rsk-dependent pathway that prevents S phase, and the other is a novel pathway that is not mediated by Rsk and that leads to prevention of the first mitotic M phase through suppression of protein synthesis of M phase cyclins. Release from such a 'dual-lock' by fertilization results in the start of the embryonic cell cycle.

**KEY WORDS:** Cell cycle, Cyclin, Fertilization, MAP kinase, p90Rsk, Starfish eggs

## INTRODUCTION

The transition from oocyte to embryo is essential for initiating embryonic development. A key event in this transition is the start of the embryonic mitotic cell cycle by fertilization. Prior to this start, the cell cycle in oocytes is generally arrested at a particular stage of meiosis dependent on the organism: prophase of meiosis I in some bivalves, metaphase of meiosis I (meta-I) in insects and tunicates, metaphase of meiosis II (meta-II) in most vertebrates, or G1 phase (pronuclear stage) after completion of meiosis II in echinoderms (Masui, 1985; Sagata, 1996). All of these meiotic arrests are released by fertilization, resulting in the initiation of the embryonic cell cycle. However, the relationship between the release from the meiotic arrest and the start of the embryonic cell cycle remains unclear. In particular, release from the meiotic arrest at stages other than G1 is complicated because it involves both completion of the remaining part of the meiotic cell cycle and initiation of the embryonic cell cycle. Thus, species using a G1 arrest are advantageous for elucidating how the embryonic cell cycle is initiated.

MAP kinase (MAPK) is likely to be generally required for many of the diverse meiotic arrests in various organisms (for reviews, see Sagata, 1996; Masui, 2000; Kishimoto, 2003). MAPK was first identified to be essential for meta-II arrest in frog eggs (Haccard et al., 1993; Kosako et al., 1994). Thereafter, involvement of MAPK was demonstrated in meta-II arrest of mouse eggs (Verlhac et al., 1996); G1 arrest of unfertilized eggs in starfish (Tachibana et al., 1997), sea urchin (Kumano et al., 2001) and jellyfish (Kondoh et al., 2006); and in meta-I arrest of sawfly eggs (Yamamoto et al., 2008). Meta-I arrest in tunicate eggs may also be regulated by MAPK

(Russo et al., 1998). In every case, inactivation of MAPK by fertilization results in release from meiotic arrest and the subsequent entry into the embryonic cell cycle. Thus, a key question is how a decrease in MAPK activity functions as a general initiator of the embryonic cell cycle, despite the diversity of meiotic arrest points. Identification of the mediators and effectors downstream of MAPK would help to elucidate this question.

In starfish (*Asterina pectinifera*; renamed *Patiria pectinifera* in 2007 – NCBI Taxonomy Browser), unfertilized mature eggs arrest at pronuclear stage (G1 phase) after completion of meiosis II. Fertilization releases the G1 arrest to initiate S phase and the following M phase, leading to the embryonic cell cycle. The starfish G1 arrest depends on the Mos-MAPK-Rsk (p90 ribosomal S6 kinase) pathway, in which Mos functions as a MAPK kinase kinase (Tachibana et al., 2000) and Rsk functions as a mediator immediately downstream of MAPK (Mori et al., 2006). During meiotic maturation, MAPK and Rsk are initially activated around metaphase of meiosis I, depending on new synthesis of Mos, and, unless fertilization occurs, MAPK and Rsk activities remain elevated until G1 arrest after completion of meiosis II. Although the physiological substrate of Rsk for the G1 arrest remains unclear, suppression of Rsk in unfertilized G1 eggs is necessary and sufficient for release from G1 arrest and entry into S phase (Mori et al., 2006). However, it is unknown whether these Rsk-suppressed eggs further progress into M phase and undergo embryonic cell cycling. Thus it remains unclear whether loss of Rsk activity is sufficient for starting the embryonic cell cycle.

Entry into M phase in fertilized starfish eggs is regulated by both cyclin A-Cdk1 and cyclin B-Cdk1 (Okano-Uchida et al., 1998). In G1-arrested starfish eggs, protein levels of cyclin A and cyclin B remain low, and fertilization triggers their accumulation. While cyclin B-Cdk1 remains inactive due to inhibitory phosphorylation of Cdk1 by Wee1 and Myt1, cyclin A-Cdk1 is activated solely by the accumulation of cyclin A. The active cyclin A-Cdk1 inactivates Wee1 and Myt1 via Plk1, resulting in the activation of cyclin B-Cdk1 and thus entry into M phase (Okano-Uchida et al., 2003; Tachibana et al., 2008). Thereafter, both cyclin A and cyclin B

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proteins are destroyed, leading to exit from M phase. Thus, accumulation of cyclin A and cyclin B is indispensable for starting the embryonic cell cycle.

In the present study, we found that in unfertilized G1 starfish eggs suppression of Rsk alone is not sufficient for cell cycle progression into M phase, even though S phase occurs, implying that a mechanism other than a decrease in Rsk activity functions to regulate the start of the embryonic cell cycle. We then investigated this, and showed that MAPK prevents entry into M phase through a pathway that is not mediated by Rsk but that leads to repression of protein synthesis of cyclin A and cyclin B. To block the start of the embryonic cell cycle in unfertilized starfish eggs, we propose a dual-lock mechanism in which there are two separate pathways downstream of MAPK: one is a Rsk-dependent pathway that leads to prevention of entry into S phase and the other is a Rsk-independent pathway that leads to prevention of entry into M phase.

## MATERIALS AND METHODS

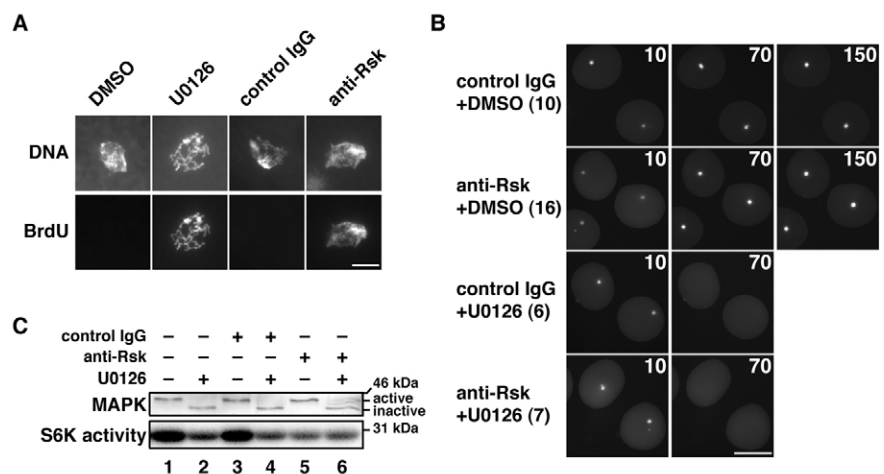
### Oocytes and eggs

Immature oocytes of the starfish *Asterina pectinifera* were prepared as described (Okano-Uchida et al., 1998). The oocytes were treated with 1  $\mu$ M 1-methyladenine (1-MeAde) to induce meiotic maturation at 20°C in artificial seawater (Jamarin Laboratory). After completion of meiosis, mature eggs with female pronuclei were inseminated. In some experiments, eggs were treated with 10  $\mu$ M U0126 (Promega), 10  $\mu$ g/ml aphidicolin (Wako) or 100  $\mu$ M emetine (Tokyo Kasei Kogyo). Egg extracts were prepared according to Okano-Uchida et al. (Okano-Uchida et al., 2003). Microinjection was performed as described (Kishimoto, 1986). Immature oocytes or unfertilized mature eggs were injected with 50 pg GST or GST-Mos (Tachibana et al., 2000); 25 pg of a fusion protein of GST, importin- $\beta$  binding domain of importin- $\alpha$  (IBB), and GFP (Tachibana et al., 2008); 3 ng purified anti-starfish Rsk antibody (Mori et al., 2006) or normal rabbit IgG (Sigma-Aldrich); 0.6 ng of a GST-tagged constitutively active form of mouse Rsk2 (CA-Rsk-EE) or its kinase negative form (KD-Rsk-EE) (Perdiguerro et al., 2003; Mori et al., 2006); 20 fmol of morpholino antisense oligonucleotides (Gene Tools) against either cyclin A (5'-GGTTTTCAGTGAAGCGAAGGACAT-3') or cyclin B (5'-ATTGTA-ACCAATGCGAGTTCTGAGG-3'); or 5 ng of peptide containing the D-box of starfish cyclin B (KSTLGTGALLENISNVAKNNV-QAAAKKEIYC) and a D-box mutant (KSTLGTAGAAENISNVA-NNVQAAAKKEIYC) (Kraft et al., 2005).

### Fig. 1. Inactivation of Rsk in G1-arrested starfish eggs leads to S phase, but further progression into M phase requires inactivation of MAPK. (A) Inhibition of either MAPK or Rsk causes DNA replication.

Unfertilized mature eggs were treated with U0126 or control DMSO, or injected with a neutralizing antibody against Rsk (anti-Rsk) or control IgG. Forty minutes later, eggs were fixed and inspected for DNA replication by incorporation of BrdU. DNA was stained with DAPI. Scale bar: 10  $\mu$ m. (B) Inhibition of MAPK causes NEBD, but inhibition of Rsk does not. Unfertilized mature eggs were injected with a neutralizing antibody against Rsk (anti-Rsk) or control IgG, along with GST-IBB-GFP. At the indicated times (minutes) after addition of U0126 or control DMSO, NEBD was scored by the dispersal of GST-IBB-GFP from the nucleus.

The number of eggs examined is indicated in parentheses. Scale bar: 200  $\mu$ m. (C) U0126 inhibits the activities of both MAPK and Rsk, but the Rsk antibody inhibits only Rsk, not MAPK. Unfertilized mature eggs were injected with a neutralizing antibody against Rsk (anti-Rsk) or control IgG, and/or treated with either U0126 or control DMSO. Sixty minutes after applying U0126 or DMSO, extracts were prepared for immunoblotting with anti-MAPK. Upper and lower bands of MAPK correspond to the active and inactive forms, respectively. Rsk activity in the same extracts was measured by phosphorylation of GST-S6 (S6K activity).



### Immunoblots

Samples were separated with 10% SDS-PAGE and transferred to Immobilon polyvinylidene fluoride membranes (Millipore). Primary antibodies used were anti-starfish cyclin A, anti-starfish cyclin B (Okano-Uchida et al., 1998), anti-MAPK (Upstate), anti-PSTAIR for Cdk1 (gift from Drs M. Yamashita and Y. Nagahama, National Institute for Basic Biology, Okazaki, Japan), anti-Phospho-Cdc2 (Tyr15; Cell Signaling Technology) and anti-GST (Nacalai Tesque). Secondary antibodies were HRP-conjugated anti-rabbit IgG (GE Healthcare) or anti-mouse IgG (Dako), AP-conjugated anti-rabbit IgG (Dako) or anti-mouse IgG (Dako), or ExactaCruzF (Santa Cruz Biotechnology). Reacted proteins were detected by the BCIP/NBT phosphatase substrate system (KPL), or ECL plus (GE Healthcare) and visualized with LAS1000-plus (Fuji Film). To reprobe with another antibody, immunoblotted membranes were stripped with WB Stripping Solution Strong (Nacalai Tesque).

### Kinase assay

Histone H1 kinase activity of whole egg extracts, or cyclin A- or cyclin B-associated Cdk1, was assayed as described (Okano-Uchida et al., 2003). Rsk activity was measured as total S6 kinase activity: 1  $\mu$ l of egg extract equivalent to half of an egg was added to 9  $\mu$ l of assay dilution buffer (25 mM Na- $\beta$ -glycerophosphate, 5 mM EGTA, 1 mM Na-orthovanadate, 1 mM DTT, 20 mM MOPS, pH 7.2), containing 16.9  $\mu$ M MgCl<sub>2</sub>, 0.1 mg/ml GST-S6 (Mori et al., 2006), Inhibitor Cocktail (20-116; Upstate) and 0.2 mCi/ml [ $\gamma$ -<sup>32</sup>P]ATP (GE Healthcare). The mixture was incubated for 10 minutes at 30°C, then 10  $\mu$ l of 2 $\times$  Laemmli sample buffer (LSB) was added, and samples were heated. Samples of histone H1 or total S6 kinase assay were separated with 12.5% or 15% SDS-PAGE, respectively, stained with Coomassie Brilliant Blue, and visualized with BAS2000 (Fuji Film).

### Immunofluorescence and live cell imaging

To assess DNA replication, eggs were incubated in seawater containing 1 mM BrdU. The vitelline coat was removed from eggs with Ca<sup>2+</sup>-free seawater, pH 9.0, containing 1% thioglycolate and 5 mM EGTA, and the eggs were treated with an extraction buffer (25 mM imidazole, 10 mM KCl, 10 mM EGTA, 1% Triton X-100, 15% glycerol, pH 6.9). The extracted eggs were attached to Biobond-coated (British BioCell) coverslips and fixed with cold methanol. After DNA denaturation with 1 M HCl, BrdU was stained with anti-BrdU (BD Biosciences) and Alexa Fluor 568 goat anti-mouse IgG (Invitrogen). DNA was stained with DAPI. For assaying nuclear envelope breakdown (NEBD), eggs were injected with GST-IBB-GFP. Fluorescence images were taken using a Zeiss AxioPlan2 microscope with Plan-Apo

40×/0.95 Korr, Plan-Apo 20×/0.60, or Plan-Apo 10×/0.32 Ph1 objectives and an AxioCam camera at room temperature (~20°C). The images were acquired with AxioVision (Zeiss) and processed in Photoshop (Adobe).

### <sup>35</sup>S Pulse-labeling of eggs

Ten eggs were pulse-labeled with 360 μCi/ml of Redivue Pro-mix L-[<sup>35</sup>S] in vitro Cell Labeling Mix (GE Healthcare) in seawater for 5 minutes, recovered in 3 μl seawater and immediately frozen in liquid nitrogen. To measure label uptake into eggs, ten pulse-labeled eggs were washed three times with seawater and dissolved in LSB. Radioactivity was measured by liquid scintillation counter (Lapasset et al., 2008). To examine the label incorporation into cyclin A and B proteins, 7 μl of lysis buffer (160 mM Na-β-glycerophosphate, 40 mM EGTA, 30 mM MgCl<sub>2</sub>, 200 mM KCl, 200 mM sucrose, 1 mM DTT, 0.5% NP-40, 1 mM Na-orthovanadate, 25 mM NaF, pH 7.3) was added, and egg extracts were prepared according to Okano-Uchida et al. (Okano-Uchida et al., 2003). Cyclins A and B were immunoprecipitated with 10 μl of a 50% slurry of Protein A Sepharose CL-4B (GE Healthcare) conjugated with anti-cyclin A or anti-cyclin B antibody. The immunoprecipitates were washed three times with lysis buffer and dissolved in 10 μl of 2× LSB. To examine the overall protein synthesis, recovered eggs were dissolved directly in LSB. The samples were separated with 10% SDS-PAGE, and transferred to Immobilon polyvinylidene fluoride membranes (Millipore). The membranes were dried and incorporation of radioactivity was visualized with BAS2000 (Fuji Film).

### PAT assay

To measure poly(A) tail length, total RNA of immature oocytes and eggs was isolated with Sepasol-RNA I Super (Nacalai Tesque). The PAT [poly(A) test] assay was performed with 300 ng of RNA as described (Salles and Strickland, 1999). PCR was performed with mRNA specific primer. PCR products were separated with agarose gel, stained with ethidium bromide and visualized with LAS4000 (Fuji Film).

## RESULTS

### MAPK prevents M phase in unfertilized mature starfish eggs, independently of prevention of S phase by Rsk

To investigate whether S phase is followed by M phase when Rsk is inhibited in G1 eggs, we injected a neutralizing antibody against starfish Rsk (Mori et al., 2006) into unfertilized eggs that had completed meiosis and were arrested at G1 phase. S phase was

evaluated by BrdU incorporation into newly synthesized DNA and M phase was evaluated by nuclear envelope breakdown (NEBD), as detected by whether a fluorescent protein with a nuclear localization sequence (IBB-GFP; see Materials and methods) was localized in the nucleus or dispersed within the cytoplasm. As shown in Fig. 1, inhibition of Rsk initiated DNA synthesis, as we reported previously (Mori et al., 2006), but did not stimulate NEBD. Thus, in G1 phase-arrested eggs, loss of Rsk activity is sufficient for entry into S phase, but not for further cell cycle progression into M phase.

Since MAPK remained active in the Rsk-inhibited eggs (Fig. 1C), we next examined the effect of MAPK inhibition on cell cycle release from the G1 phase arrest. In contrast to the case of inhibition of Rsk alone, when the MEK inhibitor U0126 was added to unfertilized eggs arrested at G1 phase, both MAPK and Rsk were inactivated (Fig. 1C), and the eggs not only entered S phase but also progressed into M phase, in the absence of fertilization (Fig. 1A,B). Injection of the neutralizing anti-Rsk antibody before applying U0126 did not affect this result (Fig. 1B). When Rsk activity was maintained at an elevated level by injection with a constitutively active form of Rsk (CA-Rsk-EE) (Mori et al., 2006) (see also Fig. 4B), MAPK inactivation by U0126 caused entry into M phase but not S phase (Fig. 2A,B). Thus, loss of MAPK activity induces M phase in unfertilized eggs independent of Rsk activity.

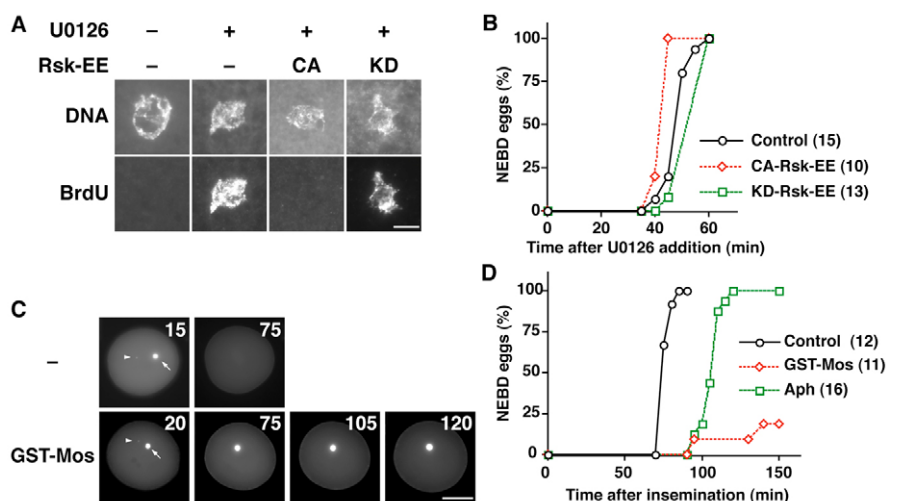
Conversely, when MAPK activity was maintained at an elevated level by prior injection of constitutively active GST-Mos, entry into M phase was prevented in fertilized eggs (Fig. 2C,D; Fig. 4C) (Mori et al., 2006; Tachibana et al., 2008). Although these eggs did not enter into S phase owing to the presence of active Rsk, the prevention of M phase did not appear to be caused by inhibition of S phase, as prevention of S phase by aphidicolin (Tachibana et al., 2008) did not inhibit entry into M phase, although some delay was observed (Fig. 2D).

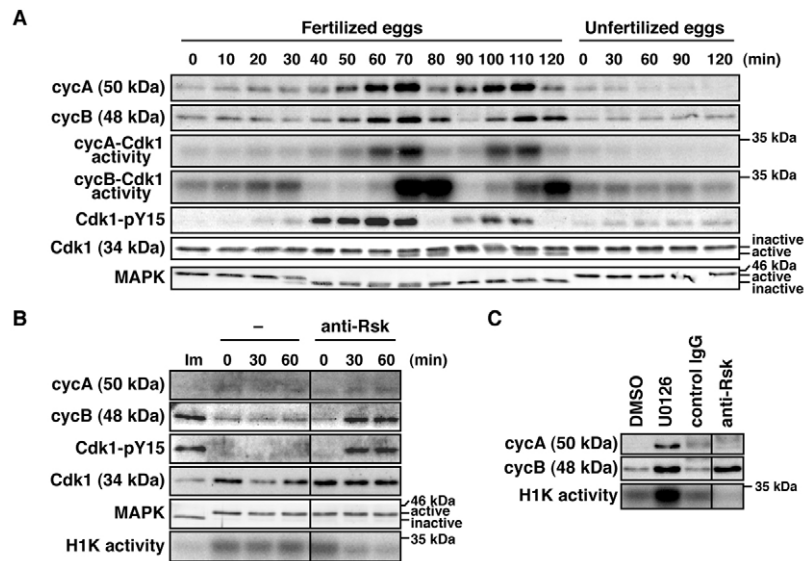
Taken together, the above observations indicate that inactivation of MAPK, but not Rsk, is necessary and sufficient for induction of M phase in unfertilized eggs, regardless of whether S phase occurs. This implies that in unfertilized mature starfish eggs arrested at G1 phase, MAPK prevents entry into M phase, independently of the Rsk-mediated prevention of entry into S phase.

### Fig. 2. Inactivation of MAPK can induce M phase in unfertilized starfish eggs in which S phase is prevented.

(A) Prevention of S phase by Rsk.

Unfertilized mature eggs were injected with either CA-Rsk-EE (CA, constitutively active form) or control KD-Rsk-EE (KD, kinase dead form), or were not injected, and were then treated with U0126 or control DMSO (-). Forty minutes later, eggs were fixed and DNA replication was detected by BrdU incorporation. DNA was stained with DAPI. Scale bar: 10 μm. (B) Entry into M phase in S phase-inhibited eggs. Unfertilized mature eggs in A were co-injected with GST-IBB-GFP. After treatment with U0126, NEBD was scored by dispersal of fluorescence of GST-IBB-GFP, as shown in Fig. 1B. The number of eggs examined is indicated in parentheses. (C,D) Requirement of MAPK inactivation for entry into M phase. Immature oocytes were injected with GST-IBB-GFP without (-) or with GST-Mos, and then treated with 1-MeAde. After completion of meiosis II, eggs were inseminated, and NEBD was examined, as in B, at the indicated times (minutes after insemination). Some eggs not injected with GST-Mos were inseminated in the presence of aphidicolin (Aph). Arrows and arrowheads indicate female and male pronuclei, respectively. Scale bar: 100 μm.





**Fig. 3. Inactivation of Rsk in unfertilized starfish eggs does not result in cyclin B-Cdk1 activation.**

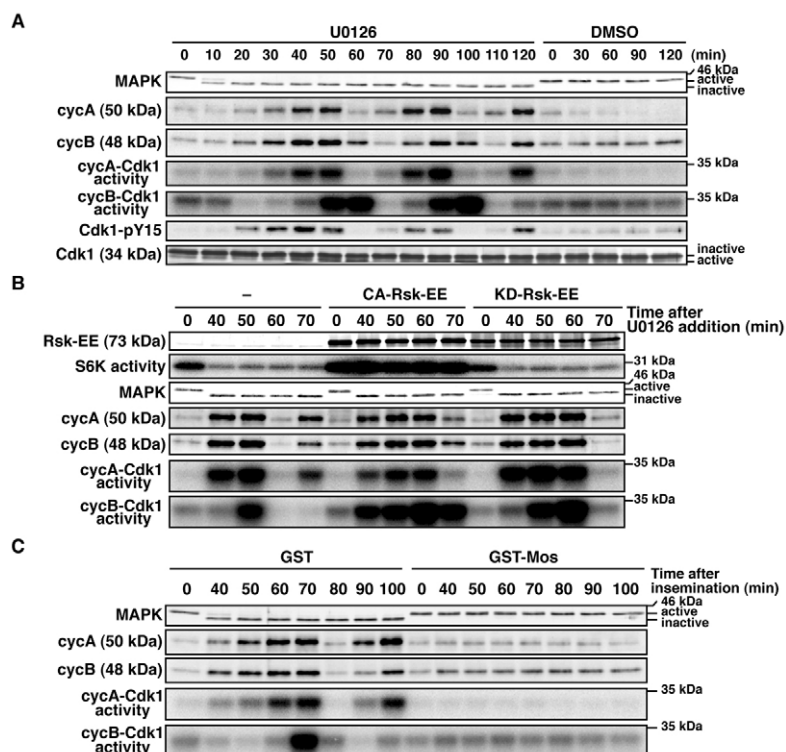
(A) Dynamics of cyclin A, cyclin B and Cdk1 after fertilization. Mature eggs arrested at G1 phase were inseminated, and egg extracts prepared at 10-minute intervals were immunoblotted with anti-cyclin A, anti-cyclin B, anti-Cdk1-pY15 and anti-PSTAIR for Cdk1, and anti-MAPK. Cdk1 activity associated with immunoprecipitates of anti-cyclin A or anti-cyclin B was measured as histone H1 kinase activity. As a control, extracts were prepared from unfertilized mature eggs at indicated times corresponding to fertilized eggs. (B,C) Dynamics of cyclin A, cyclin B and Cdk1 after Rsk inhibition. Unfertilized mature eggs were injected with a neutralizing antibody against Rsk (anti-Rsk) and recovered at indicated times (B) or at 60 minutes (C) after injection. As controls, unfertilized mature eggs that were not injected (–) were recovered at times corresponding to the injected eggs (B); alternatively, unfertilized mature eggs were either injected with control IgG or treated with U0126 or DMSO, and then recovered 60 minutes later (C). Extracts prepared from these eggs were processed for immunoblots or measurement of whole histone H1 kinase activity (H1K activity) as in A. Im, immature oocytes.

### MAPK inhibits accumulation of cyclin A and cyclin B proteins in unfertilized mature starfish eggs

To address how MAPK prevents entry into M phase, we examined the dynamics of the major cell cycle regulators for M phase, cyclins A and B and Cdk1, before and after fertilization in starfish eggs. In unfertilized mature eggs, MAPK was active, and protein levels of cyclin A and cyclin B and their associated Cdk1 activity remained low (Fig. 3A) (see Tachibana et al., 1997; Tachibana et al., 2008; Okano-Uchida et al., 1998). Fertilization led to inactivation of MAPK, accumulation of cyclin A and cyclin B proteins and activation of their associated Cdk1, peaking at ~70–80 minutes,

immediately followed by NEBD (Fig. 3A; Fig. 2D). Then, cyclin A and cyclin B proteins underwent degradation, and their associated Cdk1 became inactive, resulting in exit from M phase. Thereafter, the levels of cyclin A and B proteins and their associated Cdk1 activity oscillated along with the cleavage cycles.

When Rsk activity was suppressed by injection of its neutralizing antibody in unfertilized mature eggs, cyclin B accumulated significantly, although MAPK remained active and cyclin A did not accumulate (Fig. 3B,C; see the last section in the Results). We previously demonstrated that accumulation of cyclin A and the resulting activation of cyclin A-Cdk1 is required for activation of



**Fig. 4. Inactivation of MAPK can induce cyclin B-Cdk1 activation in unfertilized starfish eggs in which Rsk is maintained active.**

(A) MAPK inactivation results in activation of cyclin A-Cdk1 and cyclin B-Cdk1. Unfertilized mature eggs were treated with U0126 or control DMSO. Egg extracts prepared at the indicated times were immunoblotted with anti-cyclin A, anti-cyclin B, anti-Cdk1-pY15 and anti-PSTAIR for Cdk1, and anti-MAPK. Cdk1 activity associated with immunoprecipitates of anti-cyclin A or anti-cyclin B was measured as histone H1 kinase activity. (B) MAPK inactivation in the presence of Rsk activity results in activation of cyclin A-Cdk1 and cyclin B-Cdk1. Unfertilized mature eggs were uninjected (–) or injected with either CA-Rsk-EE or control KD-Rsk-EE, and then treated with U0126. Egg extracts were prepared at the indicated times. Protein levels of CA- or KD-Rsk-EE were detected by immunoblots with anti-GST. Rsk activity was assayed as S6 kinase (S6K) activity. (C) Maintenance of MAPK activity prevents cyclin A-Cdk1 and cyclin B-Cdk1 from activation induced by insemination. Immature oocytes were injected with GST-Mos or control GST, treated with 1-MeAde and then inseminated after completion of meiosis II. Egg extracts were prepared at the indicated times after insemination.

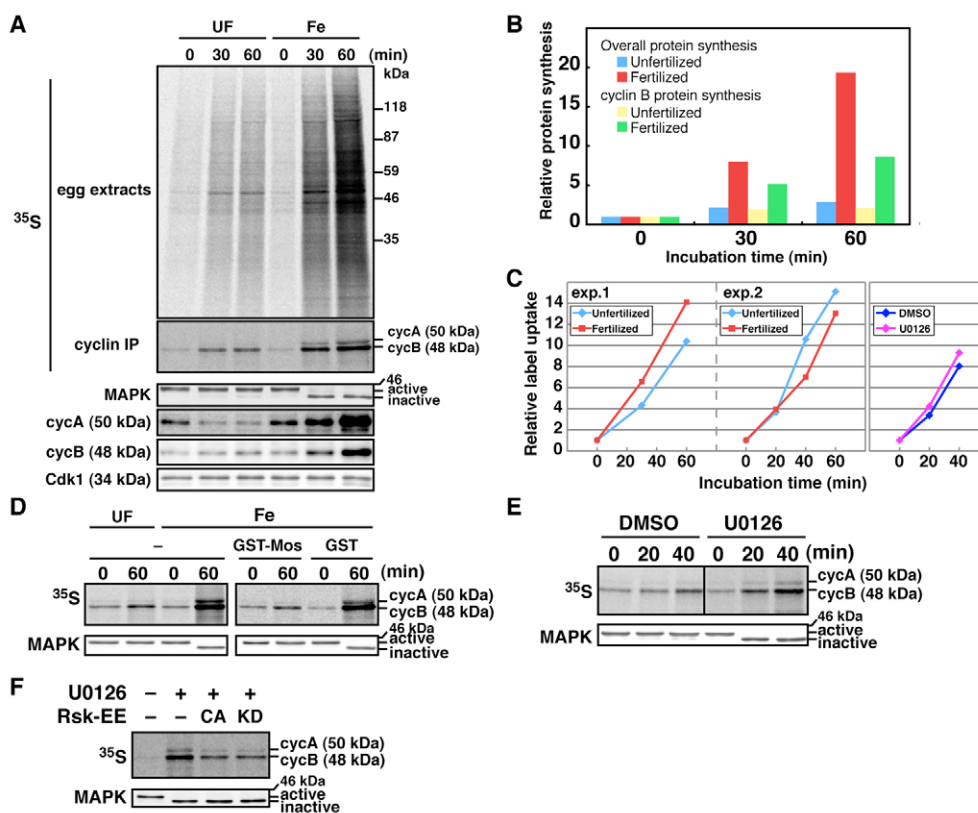
cyclin B-Cdk1 through inactivation of its inhibitory kinases, Wee1 and Myt1 (Okano-Uchida et al., 2003). This explains why, on the whole, Cdk1 was inhibited by phosphorylation on Tyr-15 and remained inactive in Rsk-inhibited eggs (Fig. 3B,C). These behaviors of Cdk1 are consistent with the above observation that inhibition of Rsk did not trigger M phase in unfertilized eggs (Fig. 1B).

By contrast, when MAPK was inactivated by U0126 in unfertilized mature eggs, both cyclin A and cyclin B accumulated to levels higher than those induced by inactivation of Rsk alone, resulting in activation of their associated Cdk1 (Fig. 4A). In these U0126-treated unfertilized eggs, cyclin A-Cdk1 was first activated, the inhibitory phosphorylation and the following dephosphorylation occurred on Cdk1-Tyr15, and finally cyclin B-Cdk1 became active. Thereafter, protein levels of cyclins A and B and their associated

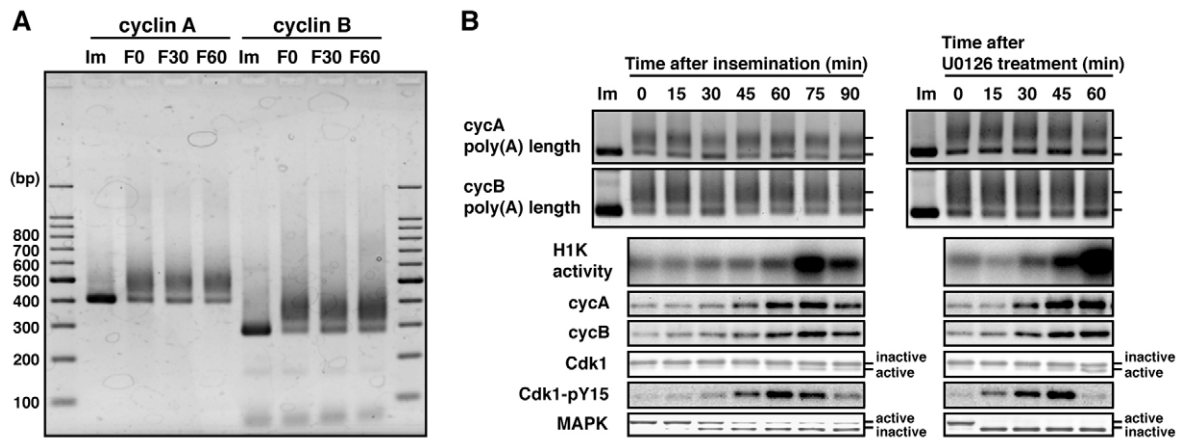
Cdk1 activity oscillated, as observed in fertilized eggs (compare Fig. 4A with Fig. 3A). Furthermore, even when Rsk activity was maintained at elevated levels by CA-Rsk-EE, MAPK inhibition by U0126 caused accumulation of cyclins A and B, resulting in activation of their associated Cdk1 (Fig. 4B; also compare with Fig. 3C). Thus, independently of Rsk, loss of MAPK activity is sufficient for accumulation of cyclins A and B and the subsequent activation of Cdk1.

Conversely, when constitutively active GST-Mos was injected into unfertilized eggs to maintain MAPK activity, neither accumulation of cyclins A and B nor activation of Cdk1 occurred when these eggs were inseminated (Fig. 4C).

Taken together, the above observations indicate that inactivation of MAPK, but not Rsk, is necessary and sufficient to cause accumulation of both cyclin A and cyclin B and the resulting



**Fig. 5. Inactivation of MAPK can induce activation of protein synthesis of cyclin A and cyclin B in unfertilized starfish eggs in which Rsk remains active.** (A,B) Incorporation of  $^{35}\text{S}$  label into overall proteins or cyclins A and B. Mature eggs were inseminated (Fe) or uninseminated (UF), and recovered at the indicated times. For 5 minutes before recovery, eggs were incubated in seawater containing  $^{35}\text{S}$ Met/Cys. Incorporation of radioactivity into overall proteins (A, top) or immunoprecipitates of cyclins A and B (A, middle) was assayed by autoradiography. Extracts were immunoblotted with anti-MAPK, anti-cyclin A, anti-cyclin B and anti-PSTAIR (A, bottom). Relative incorporation of radioactivity into overall proteins or cyclin B was measured by Multi Gauge (Fuji Film), and considered as 1 at 0 minutes (B). (C) Uptake of  $^{35}\text{S}$  label into eggs. Mature eggs with or without insemination, or U0126 treatment, were pulse-labeled with  $^{35}\text{S}$ Met/Cys as in A, washed and dissolved in LSB, followed by liquid scintillation counting. Relative uptake of radioactivity into eggs is considered as 1 at 0 minutes. (D) MAPK prevents synthesis of cyclins A and B. Immature oocytes were uninjected (-) or injected with either GST-Mos or control GST, and then treated with 1-MeAde to reinitiate meiosis. Mature eggs were inseminated (Fe) or uninseminated (UF), pulse-labeled with  $^{35}\text{S}$ Met/Cys as in A and recovered at the indicated times after insemination. Cyclins A and B were immunoprecipitated from egg extracts and incorporation of radioactivity into immunoprecipitates was assayed by autoradiography. Extracts were also immunoblotted with anti-MAPK to confirm successful insemination or effect of GST-Mos. (E) MAPK inactivation induces synthesis of cyclins A and B. Unfertilized mature eggs were treated with U0126 or control DMSO, pulse-labeled with  $^{35}\text{S}$ Met/Cys as in A, and recovered at the indicated times after U0126 addition. (F) MAPK inactivation-induced synthesis of cyclins A and B in the presence of Rsk. Unfertilized mature eggs were injected with CA-Rsk-EE (CA) or KD-Rsk-EE (KD) and then treated with U0126. Thirty-five minutes after U0126 addition, eggs were pulse-labeled with  $^{35}\text{S}$ Met/Cys as in A, and then recovered. In E and F, immunoprecipitation of cyclins A and B, autoradiography and immunoblots for MAPK were performed as in D.



**Fig. 6. Elongation of poly(A) tail in *cyclin A* and *B* mRNAs occurs during meiotic maturation but does not occur after fertilization of mature eggs. (A,B)** Total RNA was isolated either from immature oocytes (Im) or unfertilized mature eggs (F0 lane in A; 0 minute lane in B). Alternatively, mature eggs were inseminated or treated with U0126 and then total RNA was isolated at the indicated times (F30 and F60 in A and B). Poly(A) tail length was measured by the PAT assay. In B, the first embryonic cell cycle progression was confirmed by the dynamics of various cell cycle markers.

activation of both cyclin A- and cyclin B-Cdk1 in unfertilized eggs. This implies that, independently of Rsk, MAPK inhibits accumulation of cyclin A and cyclin B proteins in unfertilized mature eggs, thus preventing entry into M phase.

### MAPK, but not Rsk, represses synthesis of cyclin A and cyclin B in a poly(A)-independent manner in unfertilized mature eggs

In general, protein levels are regulated through synthesis and degradation. To address how accumulation of cyclin A and cyclin B is negatively regulated by MAPK in unfertilized mature starfish eggs, we examined the rate of synthesis of cyclin A and cyclin B in various conditions. First, we compared the rate of  $^{35}\text{S}$  label incorporation into cyclins A and B with or without fertilization by pulse-labeling de novo synthesized proteins with [ $^{35}\text{S}$ ]Met and [ $^{35}\text{S}$ ]Cys. During a 1 hour incubation, pulse-label incorporation into overall proteins was low in unfertilized mature eggs, but increased following fertilization (Fig. 5A, top; Fig. 5B). Immunoprecipitation of cyclin A and cyclin B from pulse-labeled egg extracts indicated a similar increase in label incorporation into cyclins A and B following fertilization (Fig. 5A, middle; Fig. 5B).

However, since a significant increase in pulse-label incorporation was also observed along with incubation in unfertilized eggs (Fig. 5A, UF lanes), we were suspicious of the changes in uptake of label into eggs. Indeed, a huge increase in the rate of uptake of pulse-labeling occurred along with incubation in unfertilized eggs (at present we do not know the reason), and its rate was similar in fertilized eggs (Fig. 5C, left and middle). Although it remains unclear how much of the total Met/Cys pool in eggs is formed by the labeled amino acids, comparison between the rate of label uptake into eggs and the rate of label incorporation into proteins (compare Fig. 5B with 5C) indicates that the rate of synthesis of cyclins A and B increased following fertilization.

By contrast, when MAPK was kept active by injection of GST-Mos, the increase in label incorporation into cyclins A and B following fertilization was significantly suppressed (Fig. 5D). Conversely, when MAPK was inactivated with U0126 in unfertilized eggs, a significant increase in the rate of label

incorporation into cyclins A and B was observed (Fig. 5E), whereas the rate of label uptake was similar between U0126-treated and control DMSO-treated eggs (Fig. 5C, right). Furthermore, this increase caused by MAPK inactivation was detectable even in eggs in which Rsk activity was maintained at an elevated level by injection of CA-Rsk-EE (Fig. 5F). The increase in label incorporation was slightly less than in uninjected eggs, but a similar reduction was also observed in eggs injected with kinase-dead Rsk (KD-Rsk-EE) (Fig. 5F), indicating that the reduction might be caused by CA-Rsk-EE protein itself, but not by its activity. These observations indicate that MAPK, but not Rsk, suppresses the synthesis rate of both cyclin A and cyclin B in unfertilized mature eggs.

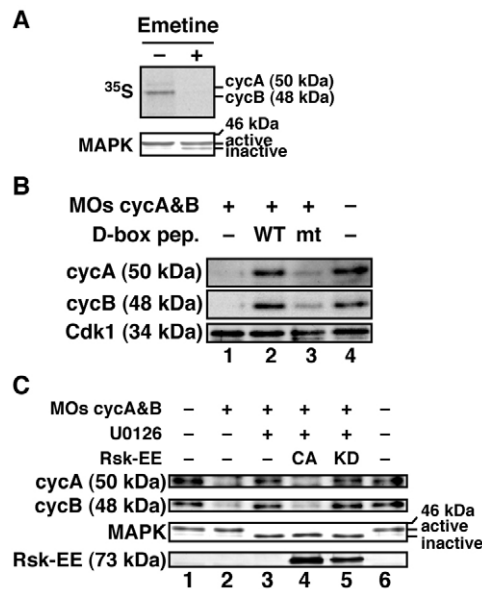
To investigate how the rate of synthesis of cyclins A and B is regulated, we performed the PAT assay (Salles and Strickland, 1999) to measure poly(A) tail length. Fig. 6 clearly shows that for both *cyclin A* and *B* mRNAs, poly(A) tail length increased during meiotic maturation but did not alter during the first cell cycle after fertilization or U0126 addition. Thus, the increase in the rate of synthesis of cyclins A and B on release from the G1 phase arrest does not appear to be regulated by elongation of poly(A) tail.

Taken together, MAPK inhibition of accumulation of cyclin A and cyclin B in unfertilized eggs is most likely to be due to poly(A)-independent repression of their synthesis rate by MAPK.

### Rsk promotes proteolysis of cyclin A and cyclin B in unfertilized mature eggs

In unfertilized mature eggs, a low but significant incorporation of [ $^{35}\text{S}$ ]Met/Cys into cyclin A and cyclin B was detectable (Fig. 5A,D,E). This incorporation was abolished by emetine, an inhibitor of protein synthesis (Fig. 7A), indicating that cyclin A and cyclin B are continuously synthesized in unfertilized eggs (even though MAPK represses the rate of synthesis). However, cyclin B protein levels were largely unaltered and cyclin A protein levels were slightly decreased in unfertilized eggs (Fig. 3A; Fig. 4A), implying that both proteins are continuously degraded in unfertilized eggs.

To confirm this, protein synthesis of cyclin A and cyclin B was specifically inhibited in unfertilized eggs. Injection of morpholino oligonucleotides (MOs) targeting *cyclin A* and *B* mRNAs resulted



**Fig. 7. Rsk promotes destruction box-dependent proteolysis of cyclin A and cyclin B in unfertilized starfish eggs.** (A) Inhibition of overall protein synthesis. Unfertilized mature eggs were incubated in the presence (+) or absence (-) of emetine for 20 minutes and then recovered. For 5 minutes before recovery, eggs were pulse-labeled with [<sup>35</sup>S]Met/Cys. Immunoprecipitation of cyclins A and B, autoradiography and immunoblots were performed as in Fig. 5D. (B) Disappearance of cyclins A and B by inhibition of their synthesis is prevented by D-box peptide. Unfertilized mature eggs were co-injected with morpholino antisense oligonucleotides (MOs) against cyclins A and B, and wild-type (WT, lane 2) or mutant (mt, lane 3) cyclin B destruction box peptide (D-box pep). As controls, eggs were uninjected (lane 4) or injected with MOs against cyclins A and B only (lane 1). After incubation for 30 minutes, eggs were recovered and the extracts were immunoblotted with anti-cyclin A, anti-cyclin B and anti-PSTAIR for Cdk1. (C) Disappearance of cyclins A and B by prevention of their synthesis requires Rsk. Unfertilized mature eggs were co-injected with CA-Rsk-EE (CA) or KD-Rsk-EE (KD) and MOs against cyclins A and B in the presence of U0126 (lanes 4 and 5). As controls, eggs were uninjected (lanes 1 and 6) or injected with MOs against cyclins A and B in the presence (lane 3) or absence (lane 2) of U0126. After incubation for 30 minutes, egg extracts were prepared and immunoblotted with anti-cyclin A, anti-cyclin B, anti-MAPK and anti-GST for CA- and KD-Rsk-EE.

in the disappearance of cyclin A and cyclin B proteins (Fig. 7B, lane 1). This disappearance was suppressed when a peptide containing the cyclin B destruction box (D-box) sequence was co-injected with the MOs, whereas co-injection of a mutant D-box peptide was not effective (Fig. 7B, lanes 2 and 3). These observations indicate that cyclin A and cyclin B undergo proteolysis in a D-box dependent manner in unfertilized eggs arrested at G1 phase.

Since inhibition of Rsk activity in unfertilized eggs resulted in the accumulation of cyclin B (Fig. 3B,C), we suspected that Rsk might promote cyclin degradation. In fact, when synthesis of both cyclin A and cyclin B was prevented by MOs, MAPK inactivation by U0126 (hence Rsk was inactivated) sustained the protein levels of both cyclins. By contrast, maintenance of Rsk activity by further injection of CA-Rsk-EE (but not KD-Rsk-EE) induced disappearance of both cyclin A and cyclin B (Fig. 7C). Thus, Rsk promoted proteolysis of cyclin A and cyclin B without MAPK activity.

Taken together, Rsk appears to positively regulate the D-box dependent proteolysis of cyclin A and cyclin B in unfertilized eggs arrested at G1 phase. It is unclear, however, why inhibition of Rsk resulted in accumulation of cyclin B, but not cyclin A, in unfertilized eggs (Fig. 3B,C; MAPK remained active). Possibly, the protein synthesis rate of cyclin A is lower than that of cyclin B (Fig. 5A,D,E), and hence accumulation of cyclin A might be below the limit of detection, even when its proteolysis is turned off by inhibition of Rsk. Alternatively, under conditions whereby Rsk activity is inhibited but MAPK remains active, an Rsk-independent pathway might support proteolysis of cyclin A in unfertilized eggs.

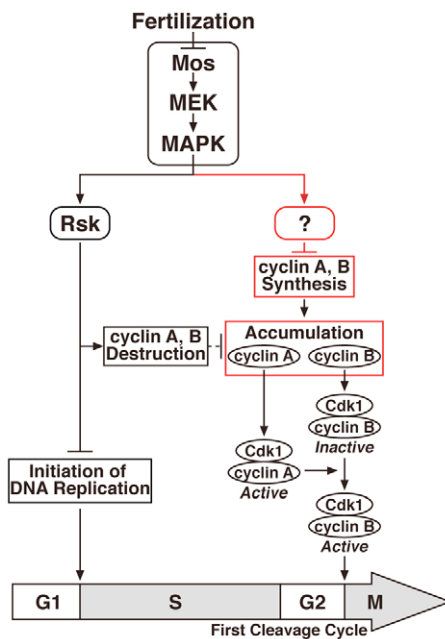
## DISCUSSION

The cell cycle arrest at G1 phase after completion of meiosis II in unfertilized mature eggs of the starfish *Asterina pectinifera* depends on Mos-MAPK (for a review, see Kishimoto, 2003). The present observations indicate that there are two separate pathways downstream of MAPK. One is the previously known pathway that is mediated by Rsk and leads to prevention of entry into S phase (Mori et al., 2006); the other is a novel pathway that is not mediated by Rsk and leads to prevention of entry into M phase through repression of protein synthesis of cyclin A and cyclin B (Fig. 8). Such a dual-lock downstream of MAPK is required for blocking the start of the embryonic cell cycle, as M phase can be initiated even when S phase is blocked, and vice versa (Figs 1 and 2). Upon fertilization, degradation of Mos shuts down both of the dual-lock mechanisms, resulting in the cell cycle progression into S phase and M phase. To our knowledge, this is the first demonstration that the Mos-MAPK cascade separates into Rsk-dependent and Rsk-independent pathways, thereby arresting the cell cycle prior to fertilization.

## Control of the embryonic cell cycle order without checkpoints

In somatic cells, the orderly progression of the cell cycle is ensured by checkpoint controls (Hartwell and Weinert, 1989). For example, the DNA replication checkpoint monitors progression of S phase (DNA replication) and allows entry into M phase only after completion of S phase. Unlike the ordinary somatic cell cycle, however, a functional cell cycle checkpoint is lacking in the early embryonic cell cycle of some organisms, including frog (Newport and Dasso, 1989), starfish and some sea urchins (Yamada et al., 1985). In starfish, when DNA replication is inhibited, the early embryonic cell cycle can progress with M phase cycling without S phase (Nagano et al., 1981) (Fig. 2). Thus, to block the start of the embryonic cell cycle in unfertilized starfish eggs arrested at G1 phase, entry into M phase must be suppressed independently of prevention of S phase, as revealed by the present study (Fig. 8).

How then is the order of S phase and M phase in the first embryonic cell cycle ensured in the absence of the DNA replication checkpoint? Since entry into M phase is controlled by the M phase cyclin-Cdk1 complex (Nurse, 1990), there are in general two ways to maintain low Cdk1 activity until the start of M phase: one is prevention of synthesis of M phase cyclin, and the other is prevention of activation of the M phase cyclin-Cdk1 complex after synthesis of M phase cyclin. In the first case, when the arrest is released, there should be a period required to accumulate M phase cyclin to a critical level necessary for entry into M phase, thereby allowing time for S phase to occur before M phase. Thus in the dual-lock mechanism (Fig. 8), the Rsk-independent pathway, which prevents Cdk1 activation before, but not after, synthesis of M phase cyclins, contributes to the ordering of the first cell cycle events after fertilization by delaying M phase initiation and thereby allowing S phase.



**Fig. 8. Dual-lock to the start of the embryonic cell cycle in unfertilized mature eggs of the starfish *Asterina pectinifera*.** Unless fertilization occurs, mature eggs of the starfish *Asterina pectinifera* arrest at the G1 phase of the pronuclear stage after completion of meiosis II. To block the start of the embryonic mitotic cycle, two separate pathways emerge downstream of the Mos-MAPK pathway. One is the established Rsk-mediated pathway that leads to prevention of entry into S phase (Mori et al., 2006). The other is a novel Rsk-independent pathway that prevents entry into M phase. Such a dual-lock downstream of MAPK is necessary for G1 arrest, as the lack of a DNA replication checkpoint allows entry into M phase in the absence of S phase. The Rsk-independent pathway causes inhibition of protein synthesis of cyclin A and cyclin B (although the direct target of MAPK remains unclear) thus preventing accumulation of both cyclins that are required for entry into M phase. Cyclin A-Cdk1 contributes to activation of cyclin B-Cdk1 via inhibition of Wee1/Myt1, inhibitory kinases for cyclin B-Cdk1 (Okano-Uchida et al., 2003). By contrast, the Rsk-dependent pathway causes destruction of cyclin B and possibly cyclin A, thus also preventing accumulation of both cyclins. However, the Rsk-dependent destruction is dispensable for prevention of entry into M phase because suppression of MAPK can induce M phase even in the presence of Rsk. In the physiological condition, fertilization induces degradation of Mos to shut down both of its downstream pathways, resulting in the cell cycle progression into S phase and M phase.

However, in the first embryonic cycle of *Xenopus*, artificial elevation of MAPK activity can cause G2 arrest (Walter et al., 1997; Bitangcol et al., 1998). In this arrest, accumulation of cyclin A and cyclin B occurs (Murakami and Vande Woude, 1998), but MAPK directly phosphorylates and activates Wee1 to inhibit Cdk1 (Murakami et al., 1999; Walter et al., 2000). Thus, MAPK-dependent repression of M phase cyclin synthesis, which is seen in starfish, might not be the only way to ensure the order of the first embryonic cycle.

### MAPK-, but not Rsk-, dependent repression of synthesis of M phase cyclins

In maturing oocytes and early embryos, protein synthesis is generally regulated through control of translational activity of maternal mRNAs because these cells are transcriptionally inert.

During oocyte maturation in starfish, synthesis of cyclins A and B is likely to be regulated in a poly(A)-dependent manner (Standart et al., 1987) (Fig. 6A). Consistently, mRNAs of cyclin A and cyclin B contain cytoplasmic polyadenylation elements [CPEs (reviewed by Mendez and Richter, 2001; Richter, 2007)] in their 3' untranslated region (see Okano-Uchida et al., 1998; Miyake et al., 2001), and the involvement of CPE-binding protein (CPEB) is suggested in cyclin translation (Lapasset et al., 2005; Lapasset et al., 2008).

By contrast, after completion of meiosis II, synthesis of cyclins A and B is likely to be regulated in a poly(A)-independent manner in starfish eggs (Fig. 6). How then does MAPK repress translation of *cyclin A* and *B* mRNAs independently of poly(A) tail elongation? In *Drosophila*, translational regulation of maternal mRNAs is well-studied before and after fertilization (reviewed by Vardy and Orr-Weaver, 2007b). It should be noted that in the PAN GU (PNG) kinase complex mutants, cyclin B synthesis is downregulated in the absence of shortening of poly(A) tail length (Vardy and Orr-Weaver, 2007a). In the wild type, however, elongation of the poly(A) tail occurs at egg activation, indicating that the MAPK-dependent translational repression in starfish G1 phase eggs is different from the repression seen in the PNG mutants. Instead, considering the constant poly(A) tail length, the 5' cap-dependent regulation (see Vardy and Orr-Weaver, 2007b) should be implicated in the MAPK-dependent repression. In the case of somatic cells of mammals, MAPK-interacting kinase (Mnk), which is a direct target of MAPK, phosphorylates eIF4E (eukaryotic initiation factor 4E) (Waskiewicz et al., 1997). This phosphorylation is thought to negatively regulate 5' cap-dependent translation (Knauf et al., 2001). Mnk is thus an interesting candidate for the Rsk-independent target of MAPK in repressing synthesis of cyclins A and B in unfertilized mature starfish eggs.

In any case, it is curious that cyclin A and cyclin B proteins accumulate at entry into meiosis II (Okano-Uchida et al., 1998) but their synthesis is repressed after completion of meiosis II and in unfertilized mature eggs (Fig. 5), whereas MAPK is fully active in every case. In starfish, the overall rate of protein synthesis is low in immature oocytes, increases upon meiosis reinitiation, remains at elevated levels until the meiosis I/II transition and then diminishes in unfertilized mature eggs (Lapasset et al., 2005; Lapasset et al., 2008). In particular, cyclin B synthesis leading up to entry into meiosis II appears to depend on MAPK in starfish [Fig. 3C in Tachibana et al. (Tachibana et al., 2000)] and on the MAPK-Rsk pathway in *Xenopus* as well (Taieb et al., 2001). It is thus likely that the MAPK-dependent repression of cyclin synthesis might be established at the end of meiosis II.

Previously, we demonstrated that parthenogenetic development into bipinnaria larvae can be induced when Rsk activation is prevented at meiosis reinitiation, and hence meiosis II is skipped (Mori et al., 2006). By contrast, Fig. 1 indicates that if the timing of Rsk inhibition is delayed until G1 phase after completion of meiosis II, entry into the first M phase does not occur, and no parthenogenesis is observed. This difference in parthenogenetic capacity might be explained by the above consideration that the MAPK-dependent suppression of protein synthesis should be established during meiosis II, although loss of centrioles, that can be duplicated, during meiosis II (Tamura and Nemoto, 2001) might also be a factor.

### Rsk-dependent M phase cyclin destruction in unfertilized starfish eggs

Meta-II arrest in unfertilized *Xenopus* eggs is accomplished by Mos-MAPK-Rsk (Sagata et al., 1989; Haccard et al., 1993; Kosako et al., 1994; Bhatt and Ferrell, 1999; Gross et al., 1999) and Erp1/Emi2



(Schmidt et al., 2005; Tung et al., 2005). Erp1 is an inhibitor of the anaphase-promoting complex/cyclosome (APC/C), which is an E3 ligase for the destruction of mitotic cyclins (Peters, 2002). Direct phosphorylation of Erp1 by Rsk stabilizes Erp1 and also promotes its APC/C-inhibiting activity, resulting in meta-II arrest (Inoue et al., 2007; Nishiyama et al., 2007). On release from meta-II arrest, Erp1 undergoes degradation that depends on Ca<sup>2+</sup>-CaMKII, Plk1 and SCF $\beta$ -TrCP, and hence the APC/C becomes active to cause degradation of cyclin B (Liu and Maller, 2005; Rauh et al., 2005). Thus, Rsk negatively regulates cyclin B destruction in *Xenopus* eggs.

By contrast, Rsk positively regulates destruction of cyclin A and cyclin B in unfertilized starfish eggs arrested at G1 (Fig. 7). How can Rsk exert opposing effects on M phase cyclin destruction in *Xenopus* and starfish eggs? Cyclin B ubiquitylation is performed by APC/C associated either with Cdc20 in M phase or Cdh1 in G1 phase (Peters, 2002). As Cdh1 protein is undetectable in unfertilized eggs and until mid-blastula stage in *Xenopus* (Lorca et al., 1998), Cdc20 is probably a major activator of APC/C in meiotic maturation and early development. The activity of APC/C associated with Cdc20 is positively regulated by Cdk1 (Kramer et al., 2000). Consistently, low but significant levels of Cdk1 activity were detectable in unfertilized starfish eggs in which Rsk was active, whereas Cdk1 became inactive after abolishment of Rsk activity (Fig. 3B) (Tachibana et al., 2008). This Cdk1 activity should be supported by Rsk, as Rsk can phosphorylate Myt1 and downregulate its inhibitory activity on cyclin B-Cdk1 (Palmer et al., 1998). Thus, Rsk could maintain APC/C-Cdc20 activity and hence promote destruction box-dependent proteolysis of M phase cyclins in unfertilized starfish eggs, assuming that Erp1 is absent or not functional in these eggs.

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