

# p90<sup>Rsk</sup> is required for G1 phase arrest in unfertilized starfish eggs

Masashi Mori, Masatoshi Hara, Kazunori Tachibana and Takeo Kishimoto\*

The cell cycle in oocytes generally arrests at a particular meiotic stage to await fertilization. This arrest occurs at metaphase of meiosis II (meta-II) in frog and mouse, and at G1 phase after completion of meiosis II in starfish. Despite this difference in the arrest phase, both arrests depend on the same Mos-MAPK (mitogen-activated protein kinase) pathway, indicating that the difference relies on particular downstream effectors. Immediately downstream of MAPK, Rsk (p90 ribosomal S6 kinase, p90<sup>Rsk</sup>) is required for the frog meta-II arrest. However, the mouse meta-II arrest challenges this requirement, and no downstream effector has been identified in the starfish G1 arrest. To investigate the downstream effector of MAPK in the starfish G1 arrest, we used a neutralizing antibody against Rsk and a constitutively active form of Rsk. Rsk was activated downstream of the Mos-MAPK pathway during meiosis. In G1 eggs, inhibition of Rsk activity released the arrest and initiated DNA replication without fertilization. Conversely, maintenance of Rsk activity prevented DNA replication following fertilization. In early embryos, injection of Mos activated the MAPK-Rsk pathway, resulting in G1 arrest. Moreover, inhibition of Rsk activity during meiosis I led to parthenogenetic activation without meiosis II. We conclude that immediately downstream of MAPK, Rsk is necessary and sufficient for the starfish G1 arrest. Although CSF (cytostatic factor) was originally defined for meta-II arrest in frog eggs, we propose to distinguish 'G1-CSF' for starfish from 'meta-II-CSF' for frog and mouse. The present study thus reveals a novel role of Rsk for G1-CSF.

**KEY WORDS:** Meiotic cell cycle, G1 arrest, Fertilization, Mos, MAP kinase, p90<sup>Rsk</sup>, Parthenogenesis, Starfish oocytes

## INTRODUCTION

The female gamete of most animals arrests in the meiotic cell cycle while awaiting fertilization, and thus parthenogenesis is prevented. Depending on the organism, this arrest occurs at the beginning of meiosis I, metaphase of meiosis I, metaphase of meiosis II (meta-II) or the pronucleus stage after the completion of meiosis II (Sagata, 1996). How each of these different meiotic cell cycle arrests is executed and whether there is a common molecular principle are key issues in cell cycle arrest.

The meta-II arrest that is observed in most of unfertilized vertebrate eggs has been most extensively studied. The activity that keeps eggs arrested in meta-II was first identified in the amphibian *Rana pipiens* and was called cytostatic factor (CSF) (Masui and Markert, 1971). Later studies established that the Mos-MAPK (mitogen-activated protein kinase) pathway is an essential core component of CSF in *Xenopus* and mouse eggs (Sagata et al., 1989; Haccard et al., 1993; Shibuya and Ruderman, 1993; Colledge et al., 1994; Hashimoto et al., 1994; Verlhac et al., 1996) (for reviews, see Sagata, 1996; Masui, 2000; Kishimoto, 2003; Tunquist and Maller, 2003). The Mos-MAPK pathway maintains the activity of cyclin B-Cdc2 kinase at an elevated level (Yamamoto et al., 2005), and thereby arrests the cell cycle at meta-II in mature eggs of frog and mouse, resulting in the prevention of parthenogenetic activation.

By contrast, unfertilized mature eggs of echinoderms, including starfish and sea urchin, are arrested at the pronucleus stage. Nonetheless, the same Mos-MAPK pathway causes the G1 phase arrest at the pronucleus stage in starfish *Asterina pectinifera* eggs

(Tachibana et al., 1997; Tachibana et al., 2000) (see Kishimoto, 2004). Mature starfish eggs lacking in Mos or MAPK activity are activated parthenogenetically in the absence of fertilization. In unfertilized sea urchin eggs, MAPK is also responsible for the G1 arrest (Carroll et al., 2000), and it is plausible that a sea urchin homolog of Mos might be present and function upstream of MAPK.

Considering the different arrest phases in frog and mouse versus starfish and sea urchin eggs, it is likely that a downstream effector of the Mos-MAPK pathway should determine the arrest phase. In *Xenopus* eggs, Rsk (p90 ribosomal S6 kinase) generates the CSF activity, immediately downstream of MAPK (Bhatt and Ferrell, Jr, 1999; Gross et al., 1999). Constitutively active forms of Rsk induce metaphase arrest independently of the activation of the Mos-MAPK pathway, while the metaphase arrest fails to occur after depletion of Rsk. In mouse eggs, however, Dumont et al. (Dumont et al., 2005) reported recently that Rsk is not involved in meta-II arrest, even though Rsk is activated during mouse meiotic cycles (Kalab et al., 1996). Mouse eggs from the triple *Rsk* knockout normally arrest at meta-II, and constitutively active mutant forms of Rsk do not restore meta-II arrest in *mos*-deficient oocytes. Thus, it is intriguing whether the G1 arrest in unfertilized starfish eggs requires Rsk activity immediately downstream of MAPK.

Here, we show that Rsk is necessary and sufficient for G1 arrest in unfertilized starfish eggs. The Mos-MAPK-Rsk pathway appears to have an ability to cause G1 arrest also in early starfish embryos. In addition, Rsk activity is required for preventing parthenogenetic activation after meiosis I. Thus, the Mos-MAPK-Rsk pathway functions in starfish eggs as a G1-CSF, by extension of the original CSF (meta-II-CSF) that was defined for meta-II arrest in frog eggs.

## MATERIALS AND METHODS

### Oocytes and eggs

Immature oocytes were isolated from the starfish *Asterina pectinifera* and were treated with 1  $\mu$ M 1-methyladenine (1-MeAde) to cause maturation as described (Tachibana et al., 1997). In some cases, mature eggs with female

Laboratory of Cell and Developmental Biology, Graduate School of Bioscience, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226-8501, Japan.

\*Author for correspondence (e-mail: tkishimo@bio.titech.ac.jp)

pronuclei were inseminated. Oocyte extracts for immunoblots and histone H1 kinase assays were prepared as described (Okano-Uchida et al., 1998). Microinjection into oocytes was performed according to Kishimoto (Kishimoto, 1986). For immunofluorescence staining, oocytes were extracted overnight at 4°C in the following buffer (25 mM imidazole, 10 mM KCl, 10 mM EGTA, 1% Triton X-100, 20% glycerol, pH 6.9), and then attached to a BioBond-coated coverslip (British Bio Cell, Cardiff, UK), followed by processing as previously described (Tachibana et al., 2000). The fluorescence images were taken using Zeiss Axioplan 2 with a 40×/Apochromat lens and a digital camera (Zeiss AxioCam).

#### Preparation of recombinant proteins

To prepare a His-tagged recombinant protein of the N-terminal kinase domain of starfish Rsk (His-RskNTD: amino acids 1-156), *NdeI* and *XhoI* sites were introduced into the cDNA of Rsk (Okumura et al., 2002) by PCR. The PCR product was digested with *NdeI* and *XhoI* and ligated into pET-21 (Novagen). The His-RskNTD protein was expressed in *E. coli* BL21 (DE3) and purified with His-Bind Resin (Novagen). To prepare a glutathione S-transferase (GST) fusion protein of S6 substrate peptide (AKRRRLSSLR), oligonucleotides (forward, 5'-CGCGGATCCGCGAAACGCCGCCGCGCC-TGAGCAGCCTGCGCGCGGAATTCCG-3'; reverse, 5'-CGGAATTC-CGCGCAGGCTGCTCAGGCGCGCGGCGTTTCGCGGATCCGCG-3') that encode the S6 substrate peptide and have the cohesive ends of *BamHI* and *EcoRI* were designed. The annealed product was ligated into the *BamHI* and *EcoRI* sites of pGEX-4T-1 (Amersham). GST-S6, GST-starfish Mos (Tachibana et al., 2000), a GST-tagged constitutively active form of mouse Rsk2, CA-Rsk-EE and its kinase negative version CA-Rsk-EE KD [both plasmids were provided by A. R. Nebreda (Perdiguero et al., 2003)] were expressed in *E. coli* and purified with glutathione-Sepharose 4B (Amersham). GST-Mos, CA-Rsk-EE and CA-Rsk-EE KD, dissolved in PBS (pH 7.4) containing 0.05% NP-40, were injected into starfish eggs at 25 pg, 600 pg and 600 pg, respectively.

#### Immunoprecipitation and kinase assay

The activity of Rsk was measured with an S6 Kinase Assay Kit (Upstate Biotechnology) either in anti-Rsk immunoprecipitates (designated as Rsk activity) or in whole cell lysates (designated as S6 kinase activity). Oocyte extracts were incubated with anti-Rsk antisera 1 and protein A Sepharose CL-4B (Amersham) for 2 hours at 4°C. Beads were washed three times with immunoprecipitation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 25 mM NaF, 0.5% NP-40], then twice with assay dilution buffer [20 mM MOPS (pH 7.2), 25 mM sodium β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT]. The kinase assay of anti-starfish Rsk immunoprecipitates was carried out for 30 minutes at 30°C in a final volume of 30 μl containing 16.9 μM MgCl<sub>2</sub>, 0.1 mg/ml GST-S6 or 40 mM S6 peptide, and 0.2 mCi/ml [<sup>γ</sup>-<sup>32</sup>P] ATP (Amersham). For total S6 kinase activity, extracts corresponding to one oocyte were incubated with GST-S6 for 10 minutes at 30°C in the presence of inhibitors of protein kinase A (PKI), PKC (PKC inhibitor peptide) and calmodulin kinase II (compound R24571). The phosphorylated GST-S6 was separated on a 15% polyacrylamide gel and stained with 0.25% Coomassie Brilliant Blue R-250. Stained gels were autoradiographed with X-ray film overnight at -80°C, and quantified by Image Gauge Ver 3.4 (Fuji Film). The phosphorylated S6 peptide was spotted on filter paper, which was then autoradiographed for quantification. The histone H1 kinase assay was performed according to Okano-Uchida et al. (Okano-Uchida et al., 2003).

#### Antibodies

Polyclonal anti-starfish Rsk antisera (1 and 2) were raised in rabbits against the His-RskNTD and were purified using a His-RskNTD-transferred membrane according to Okano-Uchida et al. (Okano-Uchida et al., 1998). Antisera against Rsk were used for immunoblot and immunoprecipitation, while the purified antibodies were injected into oocytes at 3 ng to neutralize endogenous Rsk activity. As controls for the neutralizing antibody 2, oocytes were injected with either the IgG purified from preimmune serum 2 or the blocked antibody 2 that had been pre-adsorbed with the antigen, the purified recombinant His-RskNTD, for 1 hour at 20°C. Other antibodies used were anti-starfish Myt1 (Okumura et al., 2002), anti-starfish Cdc25 (Okano-Uchida et al., 1998), anti-MAPK (Upstate), anti-active MAPK

(Cell Signalling) and anti-phospho-Tyr-15 of Cdc2 (New England BioLabs). Samples were separated on 10% polyacrylamide gels for immunoblotting.

## RESULTS

### p90<sup>Rsk</sup> is activated downstream of the Mos-MAPK pathway during starfish meiotic cycles

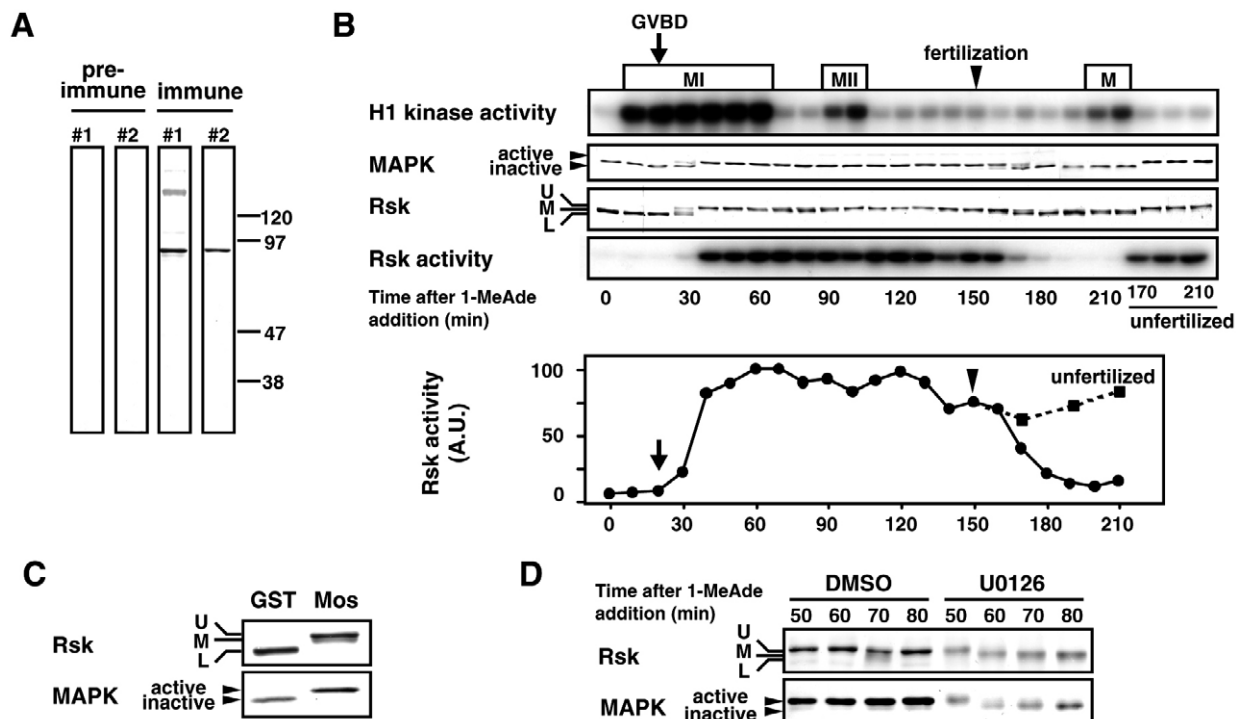
To investigate the role of Rsk in meiotic cycles in starfish oocytes, we isolated the cDNA for a starfish homolog of Rsk (Okumura et al., 2002). Only one Rsk homolog was found, as is usually the case for starfish proteins (see Kishimoto, 2003). Starfish Rsk showed ~60% amino acid identity with each of the four subtypes of mammalian Rsk. Polyclonal antibodies against a His-tagged N-terminal kinase domain of Rsk were raised in rabbits, and two kinds of antisera (1 and 2; see below) were obtained. They recognized a major band around the predicted molecular size of 83.4 kDa in extracts from immature starfish oocytes (Fig. 1A).

Immunoblots with anti-starfish Rsk antiserum revealed that protein levels of Rsk remained constant throughout meiotic and the first cleavage cycles (Fig. 1B). Precise examination of the SDS-PAGE electrophoretic mobility indicated that Rsk was detectable as a single lower band (L) in immature oocytes, as an upshifted band (U) after germinal vesicle breakdown (GVBD) until the completion of the second meiosis, and then as the middle-shifted band (M) after fertilization, while the U band remained unless fertilization occurred (Fig. 1B, third panel). In some individuals, both U and M bands were detectable after GVBD, and only the U band disappeared after fertilization (data not shown). Rsk activity measured in anti-Rsk immunoprecipitates was very low in immature oocytes, rapidly increased after H1 kinase activation and GVBD, remained at elevated levels throughout the meiotic cycles unless fertilization occurred, and then decreased at fertilization in pronucleus stage eggs (Fig. 1B, fourth and fifth panels). Thus, the U band should correspond to the active form of Rsk.

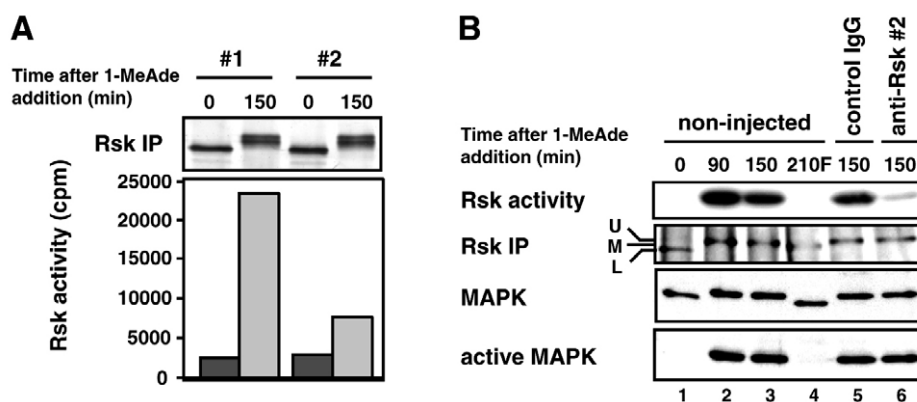
As these changes in Rsk activity correlated completely with those in MAPK activity (Fig. 1B, second panel) (see also Tachibana et al., 1997; Tachibana et al., 2000), we examined whether the activation of MAPK is required for that of Rsk. When recombinant GST-Mos protein that can activate MAPK in immature starfish oocytes (Tachibana et al., 2000) was injected into immature oocytes, Rsk underwent a mobility shift up to the U band, which implies its activation, although GVBD did not occur (Fig. 1C). Conversely, when U0126, an inhibitor of MAPK kinase, was added to maturing oocytes at metaphase of meiosis I, Rsk underwent, along with the inactivation of MAPK, a mobility shift down to the L band, which implies its inactivation (Fig. 1D). These observations indicate that Rsk activity is regulated downstream of the Mos-MAPK pathway during starfish meiotic cycles.

### Rsk is responsible for the G1 phase arrest in unfertilized starfish eggs

To address the requirement of Rsk for the G1 arrest in unfertilized starfish eggs, we manipulated Rsk activity in mature eggs using an inhibitory antibody and a constitutively active form of Rsk. For this purpose, the kinase activity of Rsk was first compared in vitro following immunoprecipitation of proteins from G1 phase-arrested eggs with two Rsk antisera (Fig. 2A). These antibodies were raised against the kinase domain and thus it was expected that they might have a neutralizing activity. Although the levels of precipitated Rsk protein were similar, the activity of Rsk immunoprecipitated with serum 2 was much lower than that with serum 1, indicating that serum 2 can inhibit the Rsk activity in vitro.



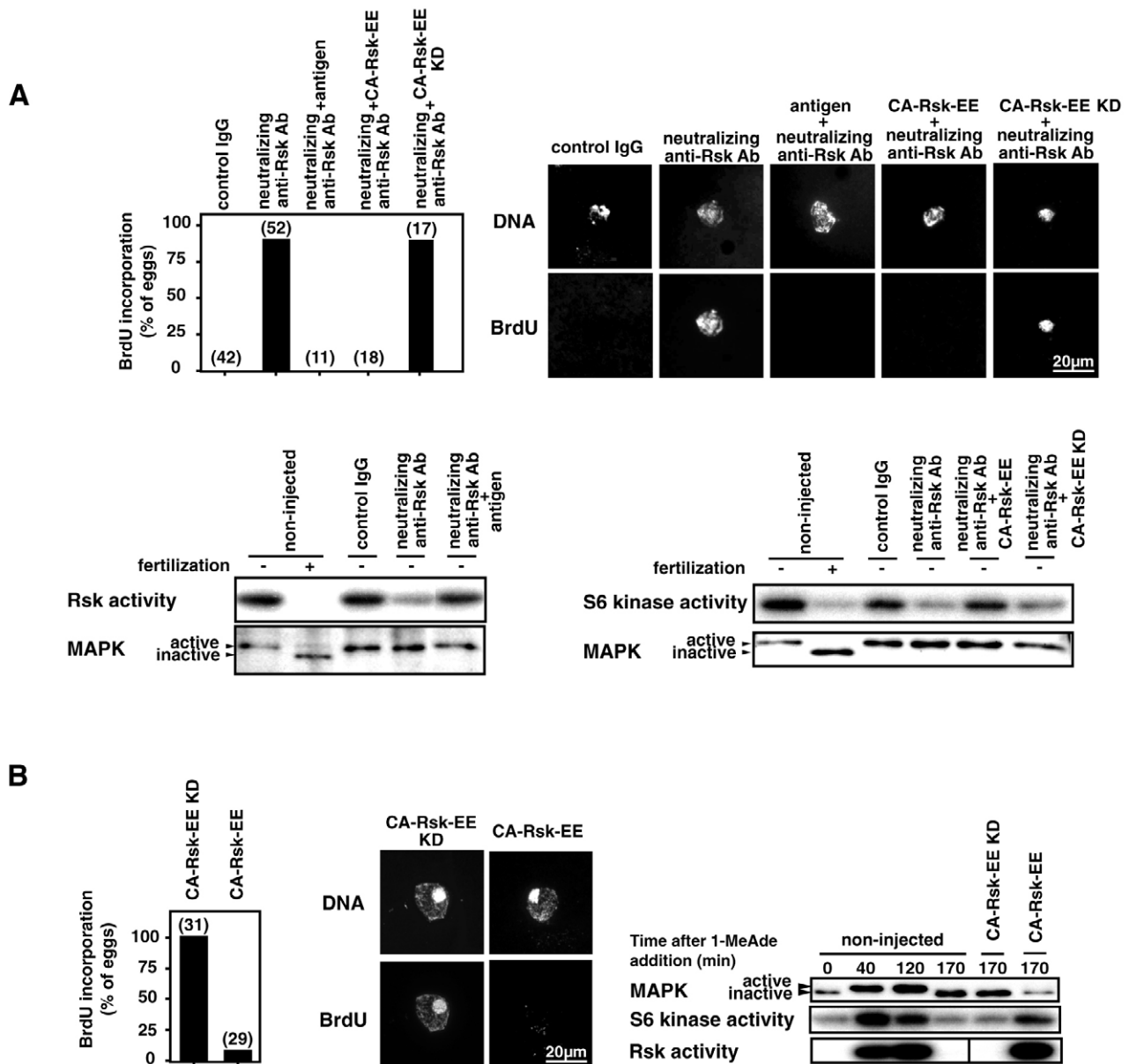
**Fig. 1. Rsk is activated downstream of Mos-MAPK pathway in starfish oocytes.** (A) Specificity of antibodies against the N-terminal kinase domain of starfish Rsk. Lysates from immature oocytes were separated on a 10% SDS-PAGE gel and blots were probed with pre-immune sera and anti-Rsk sera (1 and 2), respectively. Right side, molecular mass markers in kDa. (B) Dynamics of Rsk during starfish meiotic cycles. Extracts were prepared from oocytes and fertilized eggs at 10 minutes intervals after 1-MeAde addition, and immunoblotted with anti-MAPK antibody (second panel) and anti-Rsk serum 2 (third panel). Immunoprecipitates with the anti-Rsk serum 1 were assayed for phosphorylation of GST-S6. Rsk activity was detected on an autoradiogram (fourth panel), which was then quantified (fifth panel). Extracts were also assayed for phosphorylation of histone H1 (first panel). U, M and L indicate three forms of Rsk. The upper and lower bands of MAPK correspond to the active and inactive forms, respectively. Arrows and arrowheads indicate the time of GVBD and fertilization, respectively. (C) Activation of MAPK and Rsk by GST-Mos injection. Immature oocytes were injected with 25 pg of control GST (left) or GST-Mos (right), recovered at 60 minutes, and immunoblotted with anti-Rsk serum 2 (upper) and anti-MAPK antibody (lower). (D) Rsk inactivation by MAPK inactivation. Maturing oocytes 50 minutes after 1-MeAde addition were treated with 10  $\mu$ M U0126, an inhibitor of MAPK kinase, or control DMSO, and then recovered at 10-minutes interval. The extracts were immunoblotted with anti-Rsk serum 2 (upper) and anti-MAPK antibody (lower).



**Fig. 2. Anti-Rsk antibody 2 neutralizes Rsk activity in vitro and in vivo.** (A) The anti-Rsk serum 2 inhibits Rsk activity in vitro. Extracts were prepared from immature oocytes (0) and unfertilized mature eggs (150). Immunoprecipitates with anti-Rsk serum 1 and 2 from these extracts were immunoblotted with anti-Rsk serum 2 (upper), or assayed for phosphorylation of S6 peptide (lower). (B) The purified anti-Rsk antibody 2 inhibits Rsk activity in vivo. Immature oocytes were injected with 3 ng of the anti-Rsk antibody purified from the serum 2 (lane 6) or control IgG from preimmune serum 2 (lane 5), or not injected (lane 1-4); oocytes were then treated with 1-MeAde. Extracts were prepared at the indicated times after 1-MeAde addition, and immunoblotted with anti-MAPK antibody (third panel) and anti-active MAPK (fourth panel). Immunoprecipitates with the anti-Rsk serum 1 were immunoblotted with anti-Rsk serum 2 (second panel), and assayed for phosphorylation of GST-S6 (first panel). In lane 4 (210F), mature eggs were inseminated 150 minutes after 1-MeAde addition. In lane 6, Rsk was present in the U form but its activity was almost undetectable.

Consistently, when immature oocytes were injected with the antibody purified from serum 2 and then were treated with maturation-inducing hormone, the Rsk activity immunoprecipitated with serum 1 was almost undetectable, even though MAPK was

activated and Rsk protein was modified to the active U form (Fig. 2B). When antibody 2 was injected into mature eggs after completion of meiosis II, Rsk activity was also suppressed (see Fig. 3A). Thus, antibody 2 can neutralize the Rsk activity both in vitro and in vivo.



**Fig. 3. Rsk is necessary and sufficient for G1 arrest in starfish eggs.** (A) Inhibition of Rsk activity causes DNA replication. Unfertilized mature eggs after completion of meiosis II (120 minutes after 1-MeAde addition) were injected in the presence of BrdU with 3 ng of either the neutralizing anti-Rsk antibody 2, the control IgG from preimmune serum 2 or the control blocked anti-Rsk antibody 2. Some eggs that received the injection of the neutralizing anti-Rsk antibody 2 were further injected with 0.6 ng of CA-Rsk-EE or CA-Rsk-EE KD. Thereafter, eggs were fixed at 60 minutes incubation. DNA replication was detected by incorporation of BrdU (top panels). Lysates from these eggs were immunoblotted with anti-MAPK antibody (bottom panels). Another aliquots of the lysates were directly processed (bottom right; S6 kinase activity) or immunoprecipitated with anti-Rsk serum 1 (bottom left; Rsk activity) for phosphorylation of GST-S6. MAPK remained in the active form, even though BrdU was incorporated. As a standard, the states of MAPK, Rsk activity and S6 kinase activity were shown in mature eggs incubated for 60 minutes without (-) or with (+) fertilization. (B) Maintenance of Rsk activity prevents DNA replication. Mature eggs after completion of meiosis II were injected with 0.6 ng of CA-Rsk-EE or control CA-Rsk-EE KD, or not injected. These eggs were inseminated in the presence of BrdU, and were fixed after 50 minutes incubation. DNA replication was detected by incorporation of BrdU (left and middle). Lysates from these eggs were immunoblotted with anti-MAPK antibody (right, upper), or assayed for total S6 kinase activity (right, middle). For assay of Rsk activity by phosphorylation of GST-S6, Rsk was precipitated with anti-serum 1 for control non-injected eggs, or with glutathione-Sepharose 4B for CA-Rsk-EE or CA-Rsk-EE KD-injected eggs (right, lower). In CA-Rsk-EE-injected and inseminated eggs, MAPK was converted to the inactive form, supporting successful insemination even though DNA replication was almost undetectable. In both A and B, the numbers of eggs examined are indicated in parentheses. In the fused nucleus (B), the bright area corresponds to DNA derived from sperm nucleus, while the rest is from egg nucleus.

To determine the effect of abolishing Rsk activity, but not MAPK activity, on unfertilized mature eggs, we have examined incorporation of BrdU into DNA (Fig. 3A, top panels). In control eggs that were injected with either preimmune IgG or the blocked antibody 2, BrdU incorporation was undetectable. By contrast, it was detectable in the pronucleus of eggs that had received the injection of the neutralizing anti-Rsk antibody 2. But this effect of the neutralizing antibody was cancelled by serial co-injection with the constitutively active form of Rsk (CA-Rsk-EE), but not with its kinase inactive form (CA-Rsk-EE KD) (for these forms, see below and Fig. 3B). Consistently, Rsk activity was detectable only in anti-Rsk 1 immunoprecipitates from eggs injected with either control IgG or the blocked antibody 2 (Fig. 3A, bottom/left), and S6 kinase activity was detectable only in whole lysates from eggs that received the control IgG injection or the CA-Rsk-EE co-injection (Fig. 3A, bottom/right), while MAPK remained in the active form in all cases. These observations indicate that DNA replication was initiated by abolishing Rsk activity in the absence of both fertilization stimulus and inactivation of MAPK. Thus Rsk inactivation is sufficient for initiating DNA replication.

Conversely, a constitutively active mutant of Rsk (CA-Rsk-EE) (Perdiguero et al., 2003) was used to maintain Rsk activity. Mature eggs after completion of meiosis II were injected with either CA-Rsk-EE or a control kinase-negative form of CA-Rsk-EE (CA-Rsk-EE KD), and then were inseminated (Fig. 3B). In control eggs, both Rsk and MAPK were inactivated, and BrdU incorporation was detectable in pronucleus DNA. At the same time point, however, no BrdU incorporation was observed in CA-Rsk-EE-injected eggs in which Rsk activity, but not MAPK activity, was maintained at an elevated level. Total S6 kinase activity assay in whole lysates supports that the levels of Rsk activity in CA-Rsk-EE-injected eggs were comparable with those in unfertilized

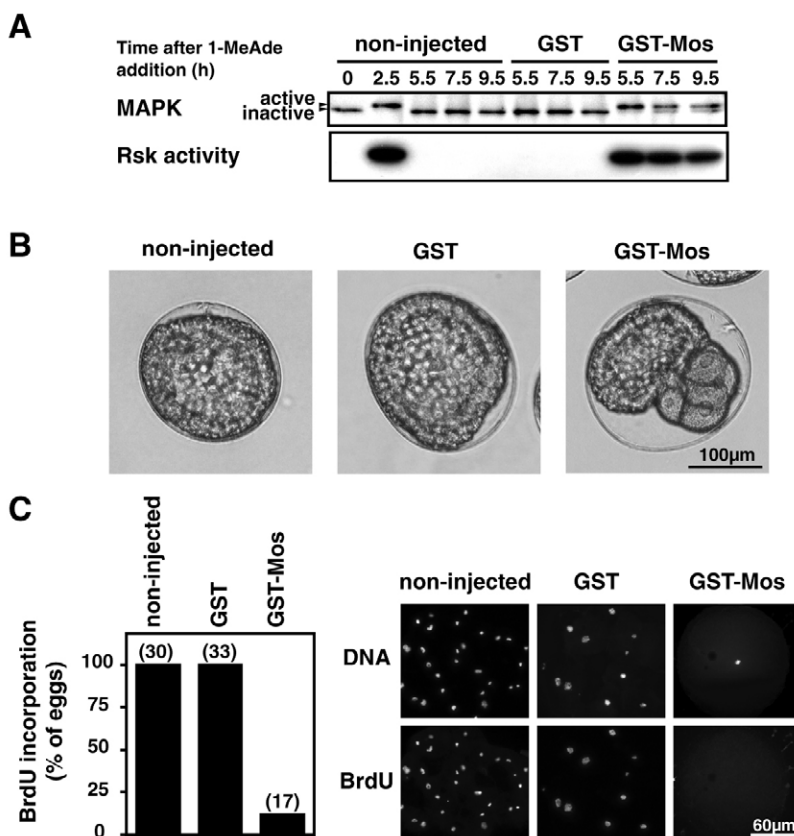
mature eggs arrested at G1 phase. Thus, inactivation of Rsk is required for initiation of DNA replication, even though MAPK is inactivated.

Taken together, these observations indicate that the only substrate of MAPK needed for G1 arrest is Rsk, and that Rsk is necessary and sufficient for G1 arrest.

### Cell cycle arrest by the Mos-MAPK-Rsk pathway during embryonic cleavage

Originally, CSF was assayed by looking for cell cycle arrest in blastomeres of frog embryos (Masui and Markert, 1971). A constitutively active form of Rsk also causes cleavage arrest at metaphase in *Xenopus* blastomeres (Silverman et al., 2004). To examine if the Mos-MAPK-Rsk pathway function as CSF by this criterion, GST-Mos was first injected into one- or two-cell stage starfish embryos after fertilization. This arrested the cell cycle in interphase, as judged by the continuous presence of the nucleus (Fig. 4B). BrdU was not incorporated into these nuclei, indicating that the arrest was at the G1 phase (Fig. 4C). Thus, Mos has a CSF-like function also in starfish embryos, though the arrest phase differs from that in frog embryos.

We did similar experiment by injecting CA-Rsk-EE. Although no cleavage occurred in blastomeres injected with CA-Rsk-EE, the arrest was observed even after control injection of CA-Rsk-EE KD (data not shown). This is possibly due to the effect of PDK1-interacting fragment (PIF) that was attached to the constitutively active form of Rsk (Perdiguero et al., 2003), as PDK1 might be involved in the regulation of cytokinesis (Bimbo et al., 2005). Nonetheless, as GST-Mos activated both MAPK and Rsk in recipient embryos (Fig. 4A), it is most likely that the Mos-MAPK-Rsk pathway has a CSF-like function through arresting starfish embryonic cycle at the G1 phase.



**Fig. 4. Mos causes G1 arrest in starfish blastomeres.**

(A,B) GST-Mos injection activates MAPK and Rsk, and causes cell cycle arrest at interphase in blastomeres. Mature eggs 2.5 hours after 1-MeAde addition were inseminated. At the two-cell stage (around 1.5-2.0 hours after insemination), one cell was injected with 25 pg of GST-Mos or control GST, or not injected, and the embryos were further cultured for 5-5.5 hours. At indicated times, lysates from whole embryos were immunoblotted with anti-MAPK antibody, or immunoprecipitated with anti-Rsk serum 1 for phosphorylation of GST-S6 (A). Whereas the non-injected half developed to the early blastula stage, nuclei were observed in the GST-Mos-injected half of large cells, indicating the cell cycle arrest at interphase (B, right). (C) GST-Mos causes G1 arrest after fertilization. Mature eggs were inseminated, and 20-30 minutes later (around entry into the first S-phase), were injected with GST-Mos or control GST, or not injected. After an additional 30 minutes (around entry into the first M-phase in control eggs), eggs were incubated for 3 hours in the presence of BrdU to examine DNA replication. In control eggs injected with GST, although significant delay in cell cycle progression was observed, BrdU was clearly incorporated into DNA. By contrast, no incorporation of BrdU was seen in GST-Mos-injected eggs, indicating cell cycle arrest at G1 phase. The numbers of eggs examined are indicated in parentheses.

## Rsk is required for prevention of parthenogenetic activation

In addition to the cell cycle arrest preceding fertilization, the Mos-MAPK pathway is required for the transition from meiosis I to meiosis II both in starfish (Tachibana et al., 2000) and *Xenopus* (Furuno et al., 1994) oocytes. It has been proposed that this second role of MAPK is conserved among invertebrate and vertebrate oocytes (Kishimoto, 2003). Downstream of the Mos-MAPK pathway, the meiosis I to II transition is also regulated by Rsk in *Xenopus* oocytes (Gross et al., 2000); by contrast, mouse oocytes do not appear to require Rsk for the meiosis I to II transition, although this has not been examined in detail (Dumont et al., 2005). To address this issue in starfish, we monitored the effect of Rsk inactivation on meiotic progression in starfish oocytes.

When immature oocytes that had been injected with the neutralizing anti-Rsk antibody were treated with maturation-inducing hormone, they underwent GVBD and emitted the first polar body normally, but no second polar body formed (Fig. 5A). These unfertilized eggs then underwent cleavage cycles resembling those in early embryonic mitotic cycles and developed parthenogenetically to bipinnaria larvae (Fig. 5B; data not shown), as seen in Mos-deficient starfish eggs (Tachibana et al., 2000).

In these oocytes lacking Rsk activity (though Rsk protein was present in the U form; see Fig. 5D, first panel), histone H1 kinase oscillated through more than two cycles after the initial activation and inactivation that correspond to meiosis I; by contrast, no H1 kinase activation occurred after meiosis II in control oocytes (Fig.

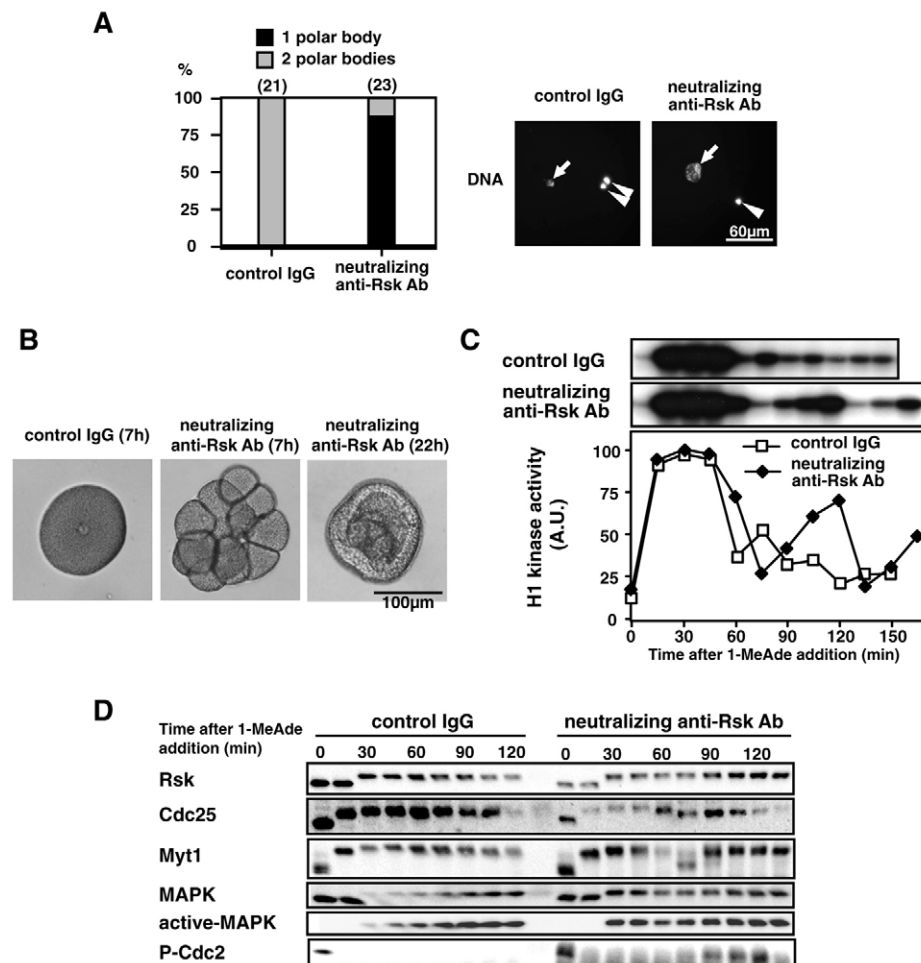
5C). Detailed analyses of Cdc2 and its regulators revealed that Cdc2 was phosphorylated on the inhibitory Tyr at the end of meiosis I, and that the levels of phosphorylation in Cdc25 and Myt1 changed in parallel with histone H1 kinase activity, in spite of the presence of active MAPK (Fig. 5D). These features in oocytes deficient in Rsk activity are in contrast to the normal meiosis I to II transition, during which Cdc25 and Myt1 remained phosphorylated, and Tyr phosphorylation of Cdc2 was undetectable.

Thus, Rsk is necessary for the transition into and the execution of meiosis II after meiosis I in starfish oocytes, as well as in *Xenopus* oocytes. Furthermore, at least in starfish oocytes, loss of such a function of Rsk appears to be sufficient to cause parthenogenetic activation after exit from meiosis I. When Rsk activity was inhibited in the pronucleus stage after completion of meiosis II, no cleavage was observed despite of occurrence of DNA replication (see Fig. 3A). It is probably due to the different states of centrioles between at the end of meiosis I and II (Uetake et al., 2002) whether cleavage occurred or not.

## DISCUSSION

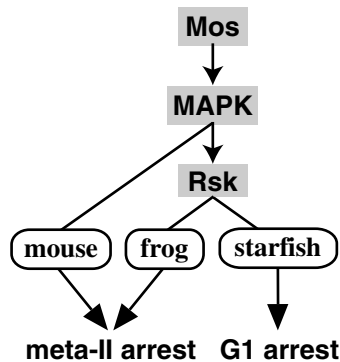
### Sufficiency of Rsk for cell cycle arrest

The present data demonstrate that Rsk is responsible for apparently all of the downstream events of the Mos-MAPK pathway in oocytes and eggs of starfish. On the one hand, this invests Rsk with a novel role for G1 arrest (Fig. 6). On the other hand, this indicates that cell cycle arrest at two different stages, G1 in starfish versus meta-II in frog, relies on an effector located on the downstream from Rsk. One



**Fig. 5. Rsk is necessary for preventing parthenogenetic activation after meiosis I in starfish oocytes.**

(A, B) Emission of only one polar body from oocytes lacking Rsk activity, followed by parthenogenesis. Immature oocytes were injected with 3 ng of control IgG or the neutralizing anti-Rsk antibody 2, and then treated with 1-MeAde. These oocytes were either fixed at 2.5 hours after 1-MeAde addition, followed by DNA staining with DAPI (A), or further incubated in the absence of insemination (B). The numbers of eggs examined are indicated in parentheses. Arrowheads indicate nucleus (A). Photographs of blastomere and gastrula were taken 7 and 22 hours, respectively, after 1-MeAde addition (B). (C) Dynamics of H1 kinase activity in oocytes lacking Rsk activity. Extracts were prepared at 15-minute intervals after 1-MeAde addition from oocytes that had been injected with 3 ng of control IgG or the neutralizing anti-Rsk antibody 2. Histone H1 kinase activity was detected on an autoradiogram (upper), which was then quantified (lower). (D) Dynamics of phosphorylation states of meiotic cell cycle regulators in oocytes lacking Rsk activity. Lysates from oocytes, that had been injected with control IgG or the neutralizing anti-Rsk antibody 2, were prepared at 15-minute intervals after 1-MeAde addition, and immunoblotted with anti-Rsk serum 2, anti-Myt1, anti-Cdc25, anti-MAPK, anti-active MAPK, anti-phospho Tyr-Cdc2 antibodies.



**Fig. 6. Meta-II-CSF versus G1-CSF.** The Mos-MAPK pathway functions as 'meta-II-CSF' in frog and mouse eggs, and as 'G1-CSF' in starfish eggs, awaiting fertilization. Rsk is an essential component for G1-CSF, but not for meta-II-CSF.

possibility is that these different phenotypes share the same effector. Alternatively, the eventual target of the Mos-MAPK-Rsk pathway could be a component of the initiation machinery of DNA replication in the starfish G1 arrest, while in the frog meta-II arrest, it could be a component of the machinery that is responsible for regulation of the APC/C (anaphase-promoting complex/cyclosome). In fact, the spindle checkpoint proteins Bub1, Mad1 and Mad2 are reported to be involved in meta-II arrest (Tunquist et al., 2002; Tunquist et al., 2003) and degradation of XErp1, an Emi1-related protein, has a key role for exit from meta-II arrest through regulation of Cdc20, the activator of the APC/C (Schmidt et al., 2005; Rauh et al., 2005).

Why then is the function of Rsk not shared between mouse and frog, even though both share the same phenotype of meta-II arrest? As suggested in Dumont et al. (Dumont et al., 2005), factors involved in spindle stability (Lefebvre et al., 2002; Terret et al., 2003) might have major roles for the mouse meta-II arrest. Nonetheless, Rsk may still have some roles for the mouse meta-II arrest, as Rsk is also activated downstream of the Mos-MAPK pathway in mouse oocytes (Kalab et al., 1996). Notably, Rsk can compensate the function of p70 S6 kinase in p70 S6 kinase-null mice (Pende et al., 2004). Even in the *Xenopus* meta-II arrest, cyclin E-Cdk2 is reported to function in parallel with the Mos-MAPK-Rsk pathway (Tunquist et al., 2002). Furthermore, in *Xenopus* eggs and their extracts, both exit from meta-II arrest (Watanabe et al., 1991) and the following DNA replication in interphase (Walter et al., 1997; Bitangcol et al., 1998) (M.M., unpublished) can occur even in the absence of inactivation of the Mos-MAPK (and possibly, -Rsk) pathway. These facts indicate that the Mos-MAPK-Rsk pathway cannot prevent release from meta-II arrest in *Xenopus*. This contrasts with the present observation in starfish that inactivation of this pathway is absolutely required for release from G1 arrest (see also Tachibana et al., 1997). It is, thus, conceivable that the sufficiency and importance of Rsk may have decreased along with evolution to higher organisms.

### Redefining CSF

CSF was originally defined as an activity that causes meta-II arrest in frog eggs (Masui and Markert, 1971; Masui, 2000). Based on the fact that its core component, the Mos-MAPK pathway, has another role in causing G1 arrest in starfish eggs, we have proposed a conceptual change in the definition of CSF (Kishimoto, 2003): CSF can be considered as a common cell cycle arrest factor to prevent

parthenogenetic activation in eggs awaiting fertilization, irrespective of the particular phase of meiotic arrest. Thus, we may distinguish 'G1-CSF' for starfish from 'meta-II-CSF' for frog and mouse (Fig. 6). According to this definition, the present study clearly indicates that G1-CSF consists of the Mos-MAPK-Rsk pathway.

We thank A. R. Nebreda for the CA-Rsk-EE constructs, K. Ohsumi and E. Okumura for suggestions, and L. A. Jaffe for critical reading of the manuscript. This work was supported by grants from the Ministry of Education, Science and Culture, Japan to K.T. and T.K., and the Human Frontier Science Program to T.K.

### References

- Bhatt, R. R. and Ferrell, J. E., Jr (1999). The protein kinase p90 rsk as an essential mediator of cytostatic factor activity. *Science* **286**, 1362-1365.
- Bimbo, A., Liu, J. and Balasubramanian, M. K. (2005). Roles of Pdk1p, a fission yeast protein related to phosphoinositide-dependent protein kinase, in the regulation of mitosis and cytokinesis. *Mol. Biol. Cell* **16**, 3162-3175.
- Bitangcol, J. C., Chau, A. S.-S., Stadnick, E., Lohka, M. J., Dicken, B. and Shibuya, E. K. (1998). Activation of the p42 mitogen-activated protein kinase pathway inhibits Cdc2 activation and entry into M-phase in cycling *Xenopus* egg extracts. *Mol. Biol. Cell* **9**, 451-467.
- Carroll, D. J., Albay, D. T., Hoang, K. M., O'Neill, F. J., Kumano, M. and Foltz, K. R. (2000). The relationship between calcium, MAP kinase, and DNA synthesis in the sea urchin egg at fertilization. *Dev. Biol.* **217**, 179-191.
- Colledge, W. H., Carlton, M. B. L., Udy, G. B. and Evans, M. J. (1994). Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs. *Nature* **370**, 65-68.
- Dumont, J., Umbhauer, M., Rassinier, P., Hanauer, A. and Verlhac, M. H. (2005). p90Rsk is not involved in cytostatic factor arrest in mouse oocytes. *J. Cell Biol.* **169**, 227-231.
- Furuno, N., Nishizawa, M., Okazaki, K., Tanaka, H., Iwashita, J., Nakajo, N., Ogawa, Y. and Sagata, N. (1994). Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes. *EMBO J.* **13**, 2399-2410.
- Gross, S. D., Schwab, M. S., Lewellyn, A. L. and Maller, J. L. (1999). Induction of metaphase arrest in cleaving *Xenopus* embryos by the protein kinase p90Rsk. *Science* **286**, 1365-1367.
- Gross, S. D., Schwab, M. S., Taieb, F. E., Lewellyn, A. L., Qian, Y.-E. and Maller, J. L. (2000). The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by p90Rsk. *Curr. Biol.* **10**, 430-438.
- Haccard, O., Sarcevic, B., Lewellyn, A., Hartley, R., Roy, L., Izumi, T., Erikson, E. and Maller, J. L. (1993). Induction of metaphase arrest in cleaving *Xenopus* embryos by MAP kinase. *Science* **262**, 1262-1265.
- Hashimoto, N., Watanabe, N., Furuta, Y., Tamemoto, H., Sagata, N., Yokoyama, M., Okazaki, K., Nagayoshi, M., Takeda, N., Ikawa, Y. et al. (1994). Parthenogenetic activation of oocytes in c-mos-deficient mice. *Nature* **370**, 68-71.
- Kalab, P., Kubiak, J. Z., Verlhac, M.-H., Colledge, W. H. and Maro, B. (1996). Activation of p90Rsk during meiotic maturation and first mitosis in mouse oocytes and eggs: MAP kinase-independent and dependent activation. *Development* **122**, 1957-1964.
- Kishimoto, T. (1986). Microinjection and cytoplasmic transfer in starfish oocytes. *Methods Cell Biol.* **27**, 379-394.
- Kishimoto, T. (2003). Cell-cycle control during meiotic maturation. *Curr. Opin. Cell Biol.* **15**, 654-663.
- Kishimoto, T. (2004). More than G1 or G2 arrest: useful starfish oocyte system for investigating skillful MAP kinase. *Biol. Cell.* **96**, 241-244.
- Lefebvre, C., Terret, M.-E., Djiane, A., Rassinier, P., Maro, B. and Verlhac, M.-H. (2002). Meiotic spindle stability depends on MAPK-interacting and spindle-stabilizing protein (MISS), a new MAPK substrate. *J. Cell Biol.* **157**, 603-613.
- Masui, Y. (2000). The elusive cytostatic factor in the animal egg. *Nat. Rev. Mol. Cell Biol.* **1**, 228-232.
- Masui, Y. and Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177**, 129-145.
- Okano-Uchida, T., Sekiai, T., Lee, K., Okumura, E., Tachibana, K. and Kishimoto, T. (1998). In vivo regulation of cyclin A/Cdc2 and cyclin B/Cdc2 through meiotic and early cleavage cycles in starfish. *Dev. Biol.* **197**, 39-53.
- Okano-Uchida, T., Okumura, E., Iwashita, M., Yoshida, H., Tachibana, K. and Kishimoto, T. (2003). Distinct regulators for Plk1 activation in starfish meiotic and early embryonic cycles. *EMBO J.* **22**, 5633-5642.
- Okumura, E., Fukuhara, T., Yoshida, H., Hanada, S., Kozutsumi, R., Mori, M., Tachibana, K. and Kishimoto, T. (2002). Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition. *Nat. Cell Biol.* **4**, 111-116.
- Pende, M., Um, S. H., Mieulet, V., Sticker, M., Gross, V. L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S. C. and Thomas, G. (2004). *SGK1<sup>-/-</sup>ISGK2<sup>-/-</sup>* mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol. Cell Biol.* **24**, 3112-3124.

- Perdiguero, E., Pillaire, M. J., Bodart, J. F., Hennersdorf, F., Frodin, M., Duesbery, N. S., Alonso, G. and Nebreda, A. R.** (2003). Xp38gamma/SAPK3 promotes meiotic G2/M transition in *Xenopus* oocytes and activates Cdc25C. *EMBO J.* **22**, 5746-5756.
- Rauh, N. R., Schmidt, A., Bormann, J., Nigg, E. A. and Mayer, T. U.** (2005). Calcium triggers exit from meiosis II by targeting the APC/C inhibitor Erp1 for degradation. *Nature* **437**, 1048-1052.
- Sagata, N.** (1996). Meiotic metaphase arrest in animal oocytes: its mechanisms and biological significance. *Trends Cell Biol.* **6**, 22-28.
- Sagata, N., Watanabe, N., Vande Woude, G. F. and Ikawa, Y.** (1989). The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* **342**, 512-518.
- Schmidt, A., Duncan, P. I., Rauh, N. R., Sauer, G., Fry, A. M., Nigg, E. A. and Mayer, T. U.** (2005). *Xenopus* polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity. *Genes Dev.* **19**, 502-513.
- Shibuya, E. K. and Ruderman, J. V.** (1993). Mos induces the *in vitro* activation of mitogen-activated protein kinases in lysates of frog oocytes and mammalian somatic cells. *Mol. Biol. Cell* **4**, 781-790.
- Silverman, E., Frodin, M., Gammeltoft, S. and Maller, J. L.** (2004). Activation of p90Rsk1 is sufficient for differentiation of PC12 cells. *Mol. Cell. Biol.* **24**, 10573-10583.
- Tachibana, K., Machida, T., Nomura, Y. and Kishimoto, T.** (1997). MAP kinase links the fertilization signal transduction pathway to the G1/S-phase transition in starfish eggs. *EMBO J.* **16**, 4333-4339.
- Tachibana, K., Tanaka, D., Isobe, T. and Kishimoto, T.** (2000). c-Mos forces the mitotic cell cycle to undergo meiosis II to produce haploid gametes. *Proc. Natl. Acad. Sci. USA* **97**, 14301-14306.
- Terret, M.-E., Lefebvre, C., Djiane, A., Rassinier, P., Moreau, J., Maro, B. and Verlhac, M.-H.** (2003). DOC1R, a MAP kinase substrate that controls microtubule organization of metaphase II arrested mouse oocytes. *Development* **130**, 5169-5177.
- Tunquist, B. J. and Maller, J. L.** (2003). Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes Dev.* **17**, 683-710.
- Tunquist, B. J., Schwab, M. S., Chen, L. G. and Maller, J. L.** (2002). The spindle checkpoint kinase Bub1 and cyclin E/Cdk2 both contribute to the establishment of meiotic metaphase arrest by cytostatic factor. *Curr. Biol.* **12**, 1027-1033.
- Tunquist, B. J., Eyers, P. A., Chen, L. G., Lewellyn, A. L. and Maller, J. L.** (2003). Spindle checkpoint proteins Mad1 and Mad2 are required for cytostatic factor-mediated metaphase arrest. *J. Cell Biol.* **163**, 1231-1242.
- Uetake, Y., Kato, K. H., Washitani-Nemoto, S. and Nemoto, S.** (2002). Nonequivalence of maternal centrosomes/centrioles in starfish oocytes: selective casting-off of reproductive centrioles into polar bodies. *Dev. Biol.* **247**, 149-164.
- Verlhac, M. H., Kubiak, J. Z., Weber, M., Geraud, G., Colledge, W. H., Evans, M. J. and Maro, B.** (1996). Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. *Development* **122**, 815-822.
- Walter, S. A., Guadagno, T. M. and Ferrell, J. E., Jr** (1997). Induction of a G2-phase arrest in *Xenopus* egg extracts by activation of p42 mitogen-activated protein kinase. *Mol. Biol. Cell* **8**, 2157-2169.
- Watanabe, N., Hunt, T., Ikawa, Y. and Sagata, N.** (1991). Independent inactivation of MPF and cytostatic factor (Mos) upon fertilization of *Xenopus* eggs. *Nature* **352**, 247-248.
- Yamamoto, T. M., Iwabuchi, M., Ohsumi, K. and Kishimoto, T.** (2005). APC/C-Cdc20-mediated degradation of cyclin B participates in CSF arrest in unfertilized *Xenopus* eggs. *Dev. Biol.* **279**, 345-355.