

Functional interaction between p90^{Rsk2} and Emi1 contributes to the metaphase arrest of mouse oocytes

Maria Paola Paronetto¹, Ezio Giorda²,
Rita Carsetti², Pellegrino Rossi¹,
Raffaele Geremia¹ and Claudio Sette^{1,*}

¹Department of Public Health and Cell Biology, Section of Anatomy, University of Rome 'Tor Vergata', Rome, Italy and ²Research Center Ospedale Bambino Gesù, University of Rome 'Tor Vergata', Rome, Italy

Vertebrate eggs arrest at metaphase of the second meiotic division before fertilization under the effect of a cytostatic factor (CSF). This arrest is established during oocyte maturation by the MAPK kinase module, comprised of Mos, MEK, MAPKs and p90^{Rsk}. Maintenance of CSF arrest at metaphase requires inhibitors of the anaphase-promoting complex (APC) like Emi1, which sequesters the APC activator Cdc20. Although it was proposed that the Mos pathway and Emi1 act independently, neither one alone is sufficient to entirely reproduce CSF arrest. Herein we demonstrate that p90^{Rsk2} associates with and phosphorylates Emi1 upstream of the binding region for Cdc20, thus stabilizing their interaction. Experiments in transfected cells and two-cell embryos indicate that Emi1 and p90^{Rsk2} cooperate to induce the metaphase arrest. Moreover, oocyte maturation was impaired by interfering with the interaction between p90^{Rsk2} and Emi1 or by RNA interference of Emi1. Our results indicate that p90^{Rsk2} and Emi1 functionally interact during oocyte maturation and that the Mos pathway establishes CSF activity through stabilization of an APC-inhibitory complex composed by Emi1 and Cdc20 before fertilization.

The EMBO Journal (2004) 23, 4649–4659. doi:10.1038/sj.emboj.7600448; Published online 4 November 2004

Subject Categories: cell cycle

Keywords: Cdc20; CSF; fertilization; metaphase arrest; mouse oocytes

Introduction

In vertebrate eggs, a cytostatic factor (CSF) is responsible for the stabilization of the cyclinB/cdc2 complex known as maturation-promoting factor (MPF) (Masui and Markert, 1971). CSF prevents the transition to anaphase until fertilization occurs and can induce cytostatic arrest when injected into the cytoplasm of mitotically dividing blastomeres (reviewed in Masui, 2001). A breakthrough in the molecular characterization of CSF came with the identification of Mos,

a serine-threonine kinase newly synthesized during oocyte maturation and capable of inducing metaphase arrest in injected blastomeres (Sagata *et al*, 1988). Mos translation during oocyte maturation establishes CSF arrest (Masui, 2001), whereas Mos depletion by RNAi prevents its establishment (Dupré *et al*, 2002). Moreover, the mitogen-activated protein kinase (MAPK) pathway is necessary for the biological activity of Mos in both *Xenopus* and mouse eggs (Haccard *et al*, 1993; Kosako *et al*, 1994; Verlhac *et al*, 2000a). The downstream effector of the Mos-dependent MAPK pathway in *Xenopus* eggs is p90^{Rsk}. It was demonstrated that p90^{Rsk2} is the predominant Rsk isoform expressed in *Xenopus* eggs and embryos and that immunodepletion of p90^{Rsk2} from cycling egg extracts completely abolished the CSF activity of Mos (Bhatt and Ferrell, 1999, 2000). Furthermore, in the absence of Mos, a CSF effect could also be obtained by microinjection of a constitutively active p90^{Rsk1} isoform (Gross *et al*, 1999), confirming that a p90^{Rsk} isoform acts downstream of Mos. The role of Mos in the establishment of cytostatic activity appears to be evolutionarily conserved in vertebrate eggs because mice with homozygous deletion of the *mos* gene ovulate oocytes that do not arrest at metaphase and undergo spontaneous parthenogenetic activation (Colledge *et al*, 1994; Hashimoto *et al*, 1994). In addition, both MAPK and p90^{Rsk} are similarly activated during meiotic maturation of mouse oocytes (Kalab *et al*, 1996; Verlhac *et al*, 1996), indicating conservation of the whole pathway.

Most of the data available on the molecular mechanisms of CSF arrest have been gathered using a constitutively active p90^{Rsk1}. Gross *et al* (2000) demonstrated that this protein inhibits the ubiquitin–ligase complex known as anaphase-promoting complex (APC), thus preventing the complete destruction of cyclin B at anaphase I and promoting its rapid accumulation during meiosis II. Recent evidence suggests that the Mos pathway inhibits the APC through activation of the spindle checkpoint (Tunquist and Maller, 2003). The kinase Bub1 is phosphorylated and activated by the constitutively active p90^{Rsk1} *in vitro* and in a MAPK-dependent manner during meiotic maturation (Schwab *et al*, 2001), and other components of this checkpoint, like Mad1 and Mad2, are required for the establishment of the Mos-dependent cytostatic arrest in *Xenopus* eggs (Tunquist *et al*, 2002, 2003). However, the function of the spindle checkpoint in CSF maintenance must be distinguishable from that exerted in mitotic cells, because the checkpoint appears active even though in metaphase-arrested oocytes chromosomes are correctly attached with their kinetochores to microtubules of the spindle. Moreover, in eggs, only Mad1 is required for maintenance of CSF, whereas Mad2, the direct inhibitor of Cdc20, is dispensable at metaphase. Thus, the connection between the endogenous p90^{Rsk} and Mad1 in CSF arrest is still unknown (Tunquist *et al*, 2003).

*Corresponding author. Department of Public Health and Cell Biology, Section of Anatomy, University of Rome 'Tor Vergata', Via Montpellier 1, 00133 Rome, Italy. Tel.: +39 06 7259 6260; Fax: +39 06 7259 6268; E-mail: claudio.sette@uniroma2.it

Received: 12 May 2004; accepted: 23 September 2004; published online: 4 November 2004

One puzzling feature of the elusive CSF of vertebrate eggs is that while the Mos/MAPK/p90^{Rsk} pathway participates in its establishment during meiosis II, it becomes dispensable afterwards. Indeed, immunodepletion of p90^{Rsk2}, or of Bub1 kinase, from *Xenopus* egg extracts is not sufficient to release them from the metaphase arrest (Bhatt and Ferrell, 1999; Tunquist *et al*, 2002). A possible explanation is that these kinases act upstream of the real effectors of the cytotstatic activity. In this regard, it has been proposed that a novel regulator of the APC, Emi1, is directly responsible of the metaphase arrest of *Xenopus* eggs (Reimann and Jackson, 2002). Emi1 interacts with the substrate-binding region of Cdc20 and stabilizes mitotic cyclins in *Xenopus* egg extracts (Reimann *et al*, 2001a, b). Moreover, immunodepletion of Emi1 from these extracts caused degradation of MPF and release from the CSF arrest, whereas an excess of Emi1 blocked release of CSF arrest by Ca⁺⁺ or activation of CamKII (Reimann and Jackson, 2002), events that mimic egg activation at fertilization (Lorca *et al*, 1993; Markoulaki *et al*, 2003). Thus, since Emi1 appeared both necessary and sufficient to produce CSF activity in *Xenopus* egg extracts, it was proposed that this protein was the long-sought CSF responsible for the metaphase II arrest of vertebrate eggs (Reimann and Jackson, 2002).

Surprisingly, the activity of Emi1 in egg extracts did not require the Mos/MAPK/p90^{Rsk} pathway, suggesting that Emi1 acts independently (Reimann and Jackson, 2002). However, it remains unclear why in the absence of Mos the cytotstatic activity of vertebrate eggs does not develop (Colledge *et al*, 1994; Hashimoto *et al*, 1994; Dupré *et al*, 2002) and why mitotic cells, which express Emi1 in the G2 phase like the oocytes, do not arrest at metaphase (Hsu *et al*, 2002). Emi1 accumulates in the S and G2 phases of mitotic cycles and its destruction at the onset of the M phase allows progression through mitosis. Phosphorylation of Emi1 by Cdc2 in prometaphase promotes its interaction with the substrate adaptor protein β TrCP and its degradation by the SCF (Skp1/Cullin/F-box) ubiquitin–ligase complex (Margottin-Goguet *et al*, 2003), dictating the timing of entry into mitosis. However, in meiosis, Emi1 is stable throughout maturation of *Xenopus* oocytes (Reimann and Jackson, 2002) but cyclin B stabilization and metaphase arrest occur only in the second division, after full activation of the Mos pathway. Mos being the only component of CSF selectively expressed during the meiotic divisions (Masui, 2001), it is possible that its pathway functionally interacts with Emi1 during maturation when CSF appears.

Herein we provide evidence that phosphorylation of Emi1 by p90^{Rsk2} stabilizes its interaction with the APC activator Cdc20 and that the two proteins cooperate to establish CSF arrest during mouse oocyte maturation. Our studies provide a direct link between components involved in the establishment and maintenance of CSF activity before fertilization.

Results

p90^{Rsk2} directly interacts with Emi1

Since p90^{Rsk2} and Emi1 are both involved in cytotstatic activity in *Xenopus* eggs, we initially investigated whether they are also present in mouse eggs. RT-PCR analysis from metaphase-arrested oocytes revealed that p90^{Rsk2} and Emi1 are both present at the mRNA level (data not shown). Both

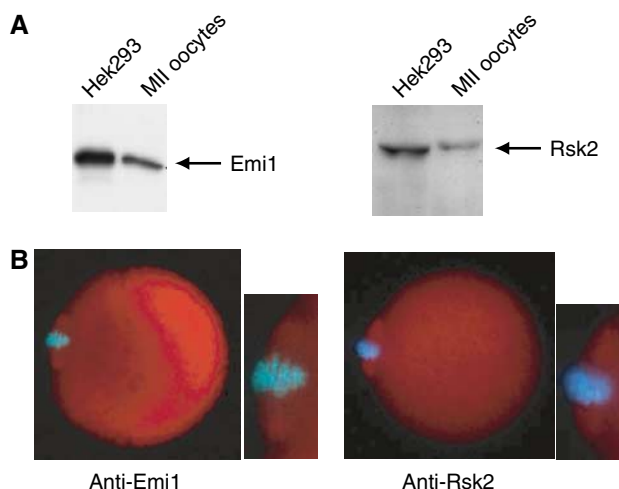


Figure 1 Emi1 and p90^{Rsk2} are expressed in mouse oocytes. (A) Western blot analysis of Emi1 and p90^{Rsk2} expression in mouse oocytes. Hek293 cell extracts (2 µg) or mouse oocyte extracts (300 oocytes) were loaded on a 10% SDS-PAGE and stained with either rabbit polyclonal anti-Emi1 antibody (1:500) or goat polyclonal anti-p90^{Rsk2} antibody (1:500). Hek293 cell extracts were loaded as positive control of protein expression. Relevant bands are indicated by arrows on the right. (B) Immunofluorescence analysis of Emi1 and p90^{Rsk2} in mouse oocytes. Double staining with either anti-Emi1 (1:200) or anti-p90^{Rsk2} (1:200) antibodies (red) and Hoechst for DNA staining (blue). Both proteins localize to the cytoplasm and the meiotic spindle (see insets).

proteins were detected in mouse oocytes by Western blot (Figure 1A) and immunofluorescence analyses showed that they were diffuse in the cytoplasm (Figure 1B). Moreover, both Emi1 and p90^{Rsk2} seemed to decorate the meiotic spindle (Figure 1B, insets), suggesting that they could partially colocalize.

Next, we investigated whether Emi1 and p90^{Rsk2} physically interact. The mouse Emi1 homologue was cloned by RT-PCR from a 13 days postcoitum (dpc) embryo library and its identity was verified by direct sequencing. HA-tagged p90^{Rsk2} and myc-tagged Emi1 were expressed in Hek293 cells either alone or in combination. When cell extracts were immunoprecipitated with anti-myc antibody, p90^{Rsk2} was specifically co-immunoprecipitated with myc-Emi1 (Figure 2A). Interestingly, we found that Emi1 also co-immunoprecipitated with the endogenous APC activator Cdc20, and that coexpression of p90^{Rsk2} increased this association (Figure 2A).

Hence, we analysed the regions of Emi1 required for this interaction using pull-down experiments with purified GST-Emi1 proteins. Emi1 contains an N-terminal region (aa 1–181 in mouse Emi1) upstream of the degradation box (F-box, aa 182–236) and a C-terminal region that contains a zinc-binding motif rich in cysteines (aa 313–383). Mitotic cyclins bind to the N-terminal region of *Xenopus* Emi1, whereas both the N-terminal and the C-terminal regions of Emi1 interact with Cdc20 (Reimann *et al*, 2001a). Remarkably, only the C-terminal region of Emi1 acts as a Cdc20 inhibitor and is necessary and sufficient for the cytotstatic activity of *Xenopus* eggs (Reimann *et al*, 2001a, b; Reimann and Jackson, 2002). We found that p90^{Rsk2} bound to full-length Emi1 and to both GST-Emi1_{1–181} and GST-Emi1_{236–383}, albeit interaction with the C-terminal region was stronger (Figure 2C). Interestingly, while the cysteine-rich motif of mouse Emi1 was required for

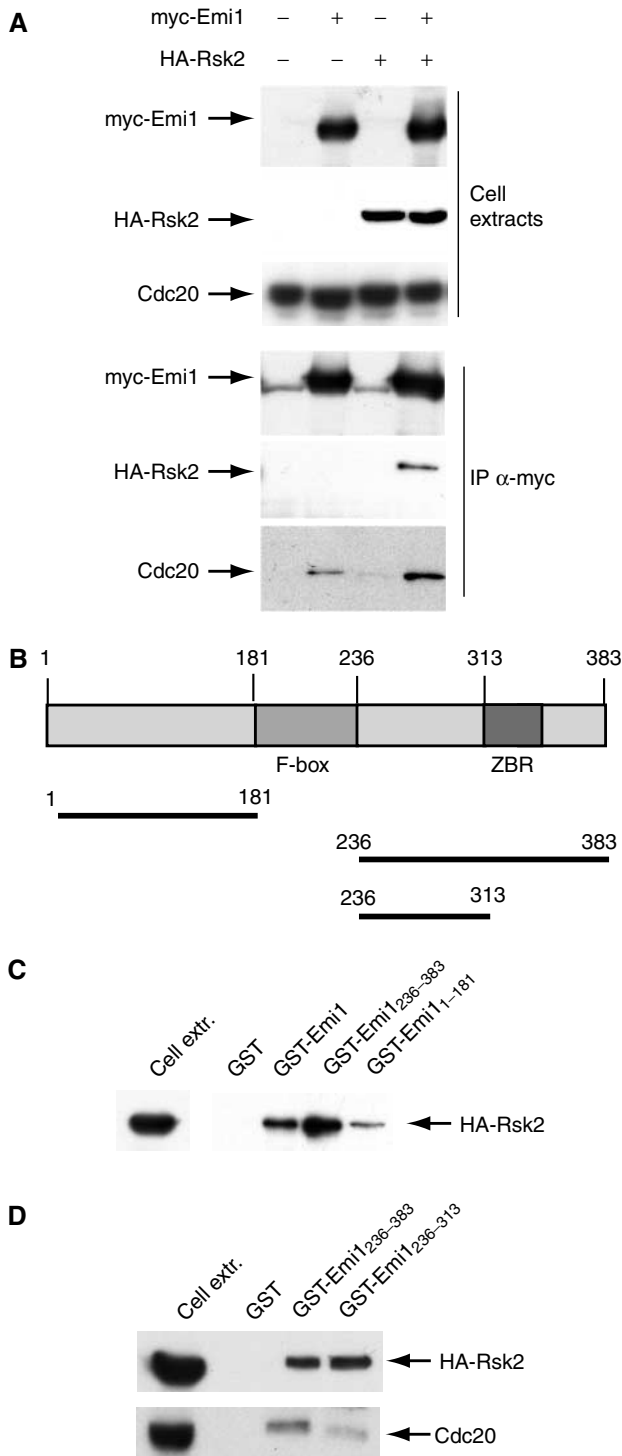


Figure 2 Emi1 associates with p90^{Rsk2}. (A) Emi1 and p90^{Rsk2} specifically co-immunoprecipitate. Western blot analyses of proteins in cell extracts (first three rows) or co-immunoprecipitating with myc-Emi1 (three bottom rows). Hek293 cells were transfected with empty vectors or myc-Emi1 and HA-p90^{Rsk2} either alone or in combination. A 20 μ g portion of total extracts was loaded in each lane (first three rows) and 500 μ g of total proteins was immunoprecipitated for each sample (last three rows). Blots were stained with anti-myc for myc-Emi1, anti-HA for HA-p90^{Rsk2}, and anti-Cdc20. (C, D) Cdc20 and p90^{Rsk2} bind to different sites in the C-terminus of Emi1. Pull-down experiments using purified GST fusion proteins of Emi1 (elucidated in the scheme in B) and extracts of Hek293 cells transfected with HA-p90^{Rsk2} (500 μ g). Bound proteins were revealed by Western blot analyses using either anti-HA or anti-Cdc20 antibodies as described in the figure.

efficient binding to Cdc20, its deletion did not affect the interaction with p90^{Rsk2} (see GST-Emi1₂₃₆₋₃₁₃ in Figure 2D), indicating that the kinase binds a different site in the C-terminus of Emi1.

p90^{Rsk2} phosphorylates Emi1 and promotes its interaction with Cdc20

To test whether Emi1 was a substrate for p90^{Rsk2}, we incubated a purified active form of p90^{Rsk2} with purified GST-Emi1 *in vitro*. As shown in Figure 3A, GST-Emi1, but not GST alone, was readily phosphorylated by the kinase. Moreover, we found that p90^{Rsk2} phosphorylated GST-Emi1₂₃₆₋₃₈₃ much more efficiently than GST-Emi1₁₋₁₈₁, and that the region of Emi1 upstream of the zinc-finger motif was sufficient for phosphorylation (GST-Emi1₂₃₆₋₃₁₃ in Figure 3B). An analysis of the known Emi1 gene sequences revealed that there are only four serine/threonine residues that are conserved between amino acids 236 and 313: ser246, thr251, thr304 and ser310 (Figure 3C). Single substitutions of these residues with

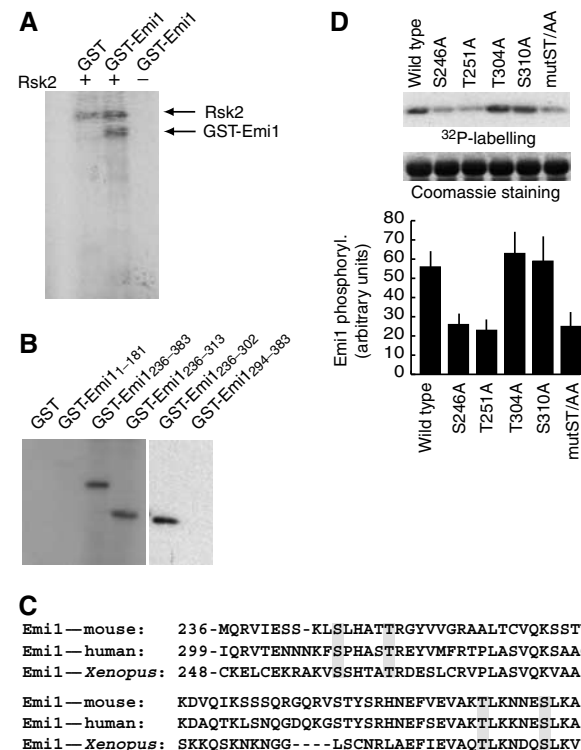


Figure 3 Emi1 is phosphorylated by p90^{Rsk2}. (A) *In vitro* kinase assay using 5 U of purified p90^{Rsk2} (Upstate Biotechnology) and 1 μ g of GST or GST-Emi1 as substrates. Proteins were incubated for 20 min at 30°C, separated on SDS-PAGE and analysed by autoradiography. (B) Emi1₂₃₆₋₃₁₃ is sufficient for phosphorylation by p90^{Rsk2}. *In vitro* kinase assay as described in (A) using purified p90^{Rsk2} and 1 μ g of the purified subregions of Emi1 described in the text of the figure. (C) Alignment of the region of homology in the 236–313 region of mouse Emi1 (NP_080271.1) reveals that only four serine/threonine residues are conserved from *Xenopus* (AAK62272) to human (AAH18905.1). (D) Site-directed mutagenesis shows that ser246 and thr251 are *in vitro* substrates for p90^{Rsk2}. The upper panel shows an *in vitro* kinase assay using purified p90^{Rsk2} and GST-Emi1₂₃₆₋₃₁₃ wild type or mutant forms. The middle panel shows the corresponding Coomassie blue staining to verify that equal amounts of GST-Emi1 proteins were used for the assay. The lower panel shows a densitometric analysis from three separate phosphorylation experiments performed as described above.

alanine showed that only ser246 and thr251 are substrates for p90^{Rsk2} (Figure 3D). Moreover, p90^{Rsk2} could still phosphorylate a GST-Emi1_{236–302} fusion protein but not GST-Emi1_{294–383} (Figure 3B). However, even when a double mutation ser246ala/thr251ala (ST/AA) was produced, phosphorylation of GST-Emi1_{236–313} was decreased but not abolished (Figure 3D), indicating that additional, nonconserved residues between amino acids 236 and 302 of Emi1 are substrates for p90^{Rsk2} *in vitro*.

Since p90^{Rsk2} interacts with and phosphorylates Emi1 upstream of the binding site for Cdc20, we asked whether it influenced their interaction. GST-Emi1 full length was bound to GSH-agarose beads and incubated for 30 min in the absence or presence of purified p90^{Rsk2} to obtain nonphosphorylated or phosphorylated sources of the protein (see Figure 3A). At the end of the incubation, beads were washed and the kinase removed, as demonstrated by Western blot analysis (third panel). Hence, beads were incubated with extracts of proliferating Hek293 cells, which express high levels of Cdc20. As expected, GST-Emi1 was able to bind to Cdc20; however, phosphorylation by p90^{Rsk2} strongly increased this interaction (approximately four-fold; Figure 4A). The same effect of phosphorylation by p90^{Rsk2} was observed when a GST-Emi1_{236–383} was used (Figure 4B), confirming that the action of p90^{Rsk2} is exerted on the region of Emi1 that is necessary and sufficient for its cytoskeletal activity (Reimann *et al*, 2001a). Remarkably, the effect of phosphor-

ylation by p90^{Rsk2} was completely suppressed when the GST-Emi1_{236–383}ST/AA mutant (ser246ala/thr251ala) was used for Cdc20 binding assays (Figure 4C). These results suggest that phosphorylation by p90^{Rsk2} stabilizes the interaction of Emi1 with Cdc20.

Emi1 and p90^{Rsk2} cooperate to induce the metaphase arrest of Hek293 cells

Since phosphorylation of Emi1 by p90^{Rsk2} increases its ability to interact with Cdc20 and this interaction prevents activation of the APC, we asked if p90^{Rsk2} could augment the cytoskeletal activity of Emi1 *in vivo*. To this end, we transfected Hek293 cells with constructs for p90^{Rsk2} and Emi1 either alone or in combination. In addition, cells were cotransfected with a GFP construct (1:10) to identify transfected cells. After 24 h from transfection, cells were fixed, stained with Hoechst and analysed for the percentage of mitotic cells in the GFP-positive population. Control cells transfected with empty vectors displayed 5% of GFP-positive cells in either prometaphase or metaphase (Figure 5A and B); overexpression of Emi1 caused an increase of mitotic cells to 12%, similar to that observed previously for *Xenopus* Emi1 (Reimann *et al*, 2001a). Interestingly, we observed that coexpression of Emi1 with p90^{Rsk2} led to a further increase in mitotic cells (24%). This effect is similar to that obtained with a stabilized Emi1 mutant (Reimann *et al*, 2001a) and suggests that the two proteins cooperate to delay or arrest Hek293 cells in mitosis.

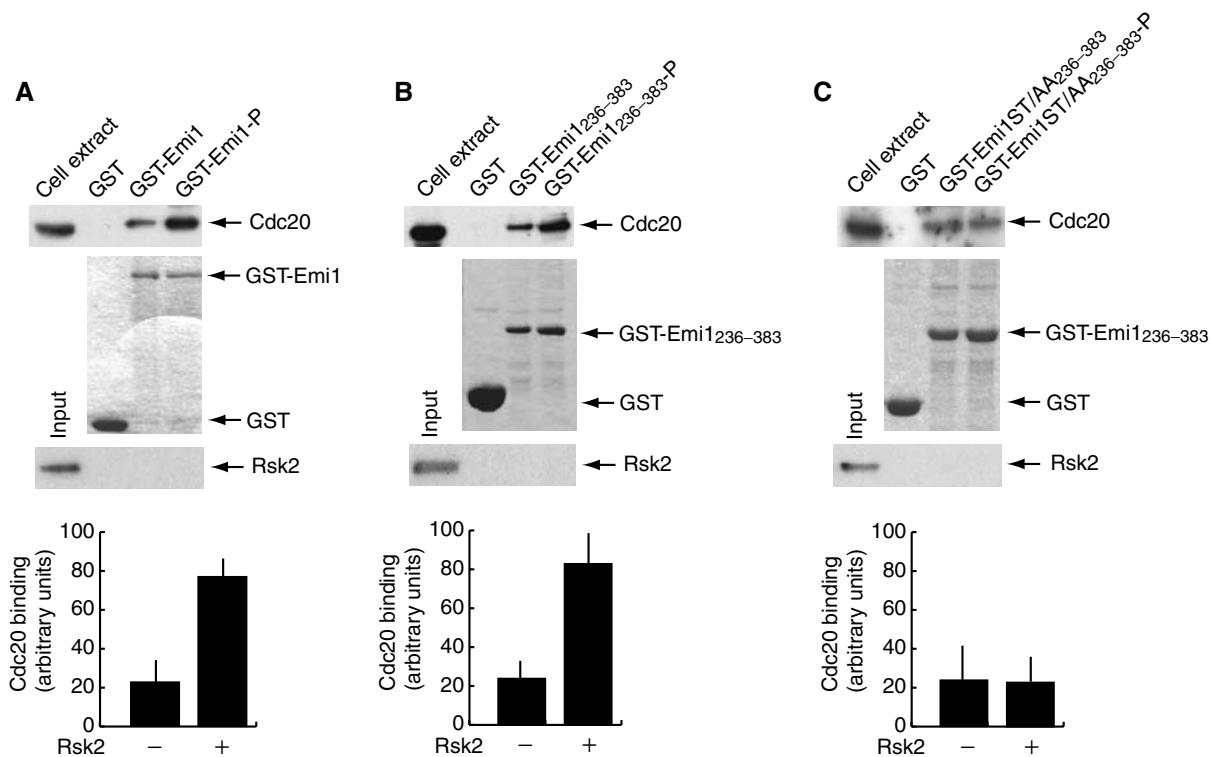


Figure 4 Phosphorylation of Emi1 by p90^{Rsk2} potentiates its binding to Cdc20. GST-Emi1 full length (A) or either wild type (B) or ST/AA mutant (C) C-terminus, aa 236–383, was purified on GSH-agarose beads and incubated for 30 min in the absence or presence of purified active form of p90^{Rsk2} to obtain a source of nonphosphorylated or phosphorylated protein. After washes to remove the kinase (the corresponding Western blot is shown in the bottom panels of each experiment), GST fusion proteins were used in pull-down experiments with extracts from Hek293 cells (500 µg of total proteins) as a source of endogenous Cdc20. The first panels in (A–C) represent anti-Cdc20 Western blot, and the second panels represent Coomassie blue staining of the pull-down experiments. The third panels display the lack of residual kinase on beads before incubation with extracts. The results from three different experiments were quantitated by optical densitometry and the data are shown as average ± standard deviation in the panels at the bottom of the figure.

Overexpression of p90^{Rsk2} alone also caused a small increase in mitotic index (9 versus 5% in control cells), which could be due to an effect of the kinase on endogenous Emi1.

To confirm that overexpression of Emi1 and p90^{Rsk2} caused a mitotic arrest in Hek293 cells, we analysed cyclin B1 expression levels and H1 kinase activity. GFP-positive and GFP-negative cells were sorted by FACS, and protein expression was tested in the two populations. Overexpression of Emi1 stabilized cyclin B1 and p90^{Rsk2} further increased the levels of cyclin B1 (Figure 5C and D). Stabilization of cyclin B1 was already appreciated when p90^{Rsk2} was transfected

alone, further suggesting an effect of p90^{Rsk2} on endogenous Emi1. Moreover, cyclin B1 increase in cells cotransfected with Emi1 and p90^{Rsk2} was accompanied by MPF activation (Figure 5C, fourth panel, and Figure 5D). These results support the hypothesis of cooperation between these proteins to induce the metaphase arrest.

Emi1 and p90^{Rsk2} cooperate to induce metaphase arrest of early blastomeres

CSF activity has been defined by the ability of purified proteins and/or extracts to induce cell cycle arrest when microinjected into dividing blastomeres. To examine the cooperation between p90^{Rsk2} and Emi1 by this classical bioassay, we microinjected these purified proteins into one blastomere of mouse two-cell embryos (Masui, 2001). We found that microinjection of GST-Emi1₂₃₆₋₃₈₃ did not exert a cytostatic effect in mouse embryos, which developed to the four-cell stage as control (not shown) or GST-injected embryos (Figure 6B). On the other hand, microinjection of an active form of p90^{Rsk2} was able to induce the typical three-cell stage arrest in 42% of microinjected embryos (Figure 6A and B). Remarkably, when p90^{Rsk2} was coinjected with GST-Emi1₂₃₆₋₃₈₃, the cytostatic effect was strongly increased (66% arrest at the three-cell stage), indicating cooperation between the two proteins *in vivo*. By contrast, GST-Emi1₂₃₆₋₃₈₃ST/AA did not reinforce the kinase action, indicating that phosphorylation on the conserved ser/thr residues of Emi1 is important for the cooperative effect (Figure 6B).

Since we found that GST-Emi1₂₃₆₋₃₁₃ is phosphorylated by p90^{Rsk2} and is sufficient for interaction with the kinase but does not interact with Cdc20, we reasoned that this portion could interfere with binding of endogenous Emi1 *in vivo* and could compete as substrate for p90^{Rsk2}. Indeed, in transfected cells, coexpression of Emi1₂₃₆₋₃₁₃ not only competed with the interaction between full-length Emi1 and p90^{Rsk2} but also prevented association of Cdc20 with the kinase (Figure 6C), suggesting that it blocks the formation of a stable complex between Emi1/Cdc20 and p90^{Rsk2}. Moreover, we found that coinjection of GST-Emi1₂₃₆₋₃₁₃ in two-cell embryos inhibited the cytostatic effect exerted by p90^{Rsk2}, with only 8% of injected embryos blocked at the three-cell stage (Figure 6A and B).

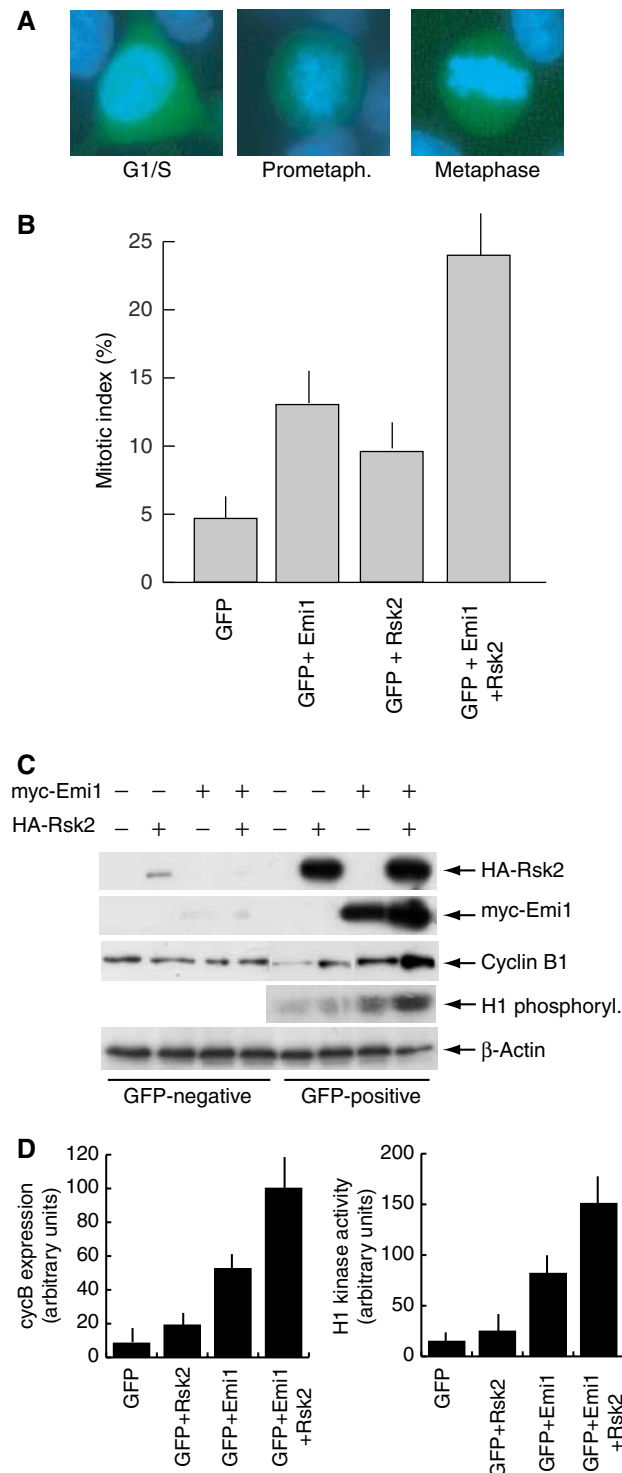


Figure 5 Emi1 and p90Rsk2 cooperate to induce cytostatic arrest in mitotic cells. Cells were transfected with either empty vector (–) or the indicated expression vector and with a GFP construct (1:10) to be able to identify transfected cells. At 24 h after transfection, cells were fixed, stained for DNA using Hoechst dye (1 µg/ml) and analysed under a fluorescence microscope to determine the percentage of mitotic cells (prometaphase and metaphase in (A) in the GFP-positive population. (B) Summary of the mitotic indexes obtained in three different experiments. In each experiment, 300 GFP-positive cells were counted in five separate fields. Data are shown as average ± standard deviation. (C) Cells transfected as described above were sorted by FACS to separate the GFP-positive population from the GFP-negative population. Cell extracts (10 µg) obtained from these populations were analysed by Western blot for HA-p90^{Rsk2}, myc-Emi1, cyclin B1 and β-actin. The first and second panels demonstrate that recombinant proteins were almost exclusively expressed in the GFP-positive population, assessing the quality of the sorting procedure. MPF activity was measured in GFP-positive cell extracts (10 µg) using H1 (1 µg) as substrate. (D) The results from three different experiments for cyclin B expression and H1 kinase activity were quantitated by optical densitometry and the data are shown as average ± standard deviation.

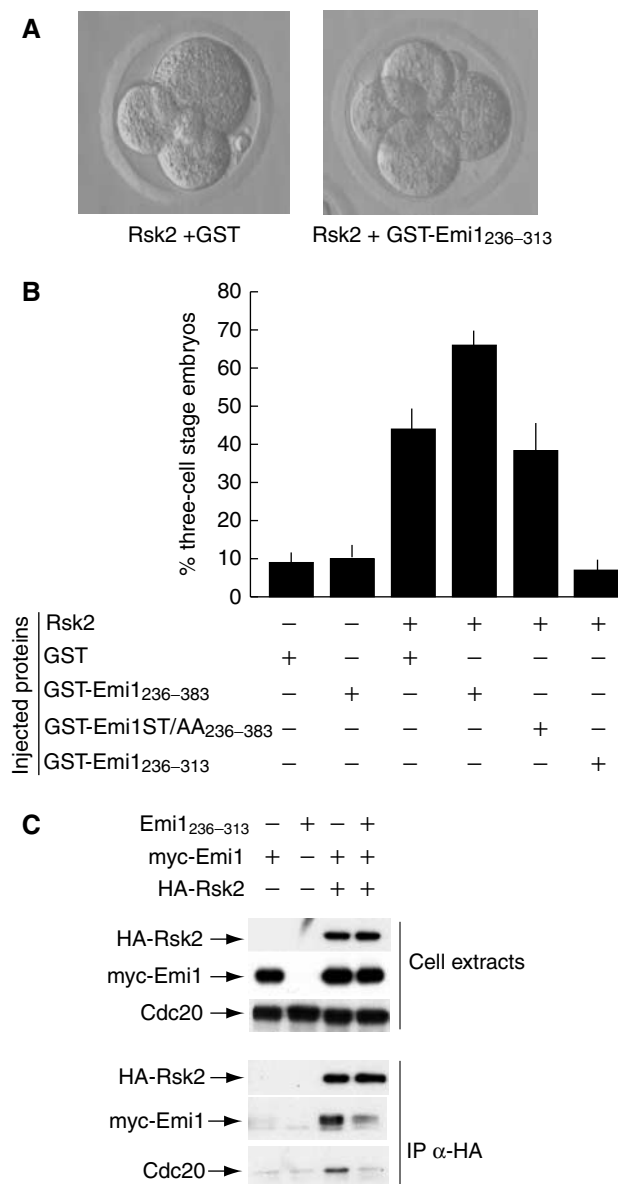


Figure 6 Emi1 potentiates the cytotstatic activity of p90^{Rsk2} in mouse blastomeres. Purified GST fusion proteins (5–8 pl of 1 mg/ml solution of GST, GST-Emi1₂₃₆₋₃₈₃ or GST-Emi1₂₃₆₋₃₁₃) were injected either alone or with purified active p90^{Rsk2} in one blastomere of two-cell mouse embryos (45–50 h after hCG injection). At 12–15 h after microinjection, embryos were scored for cell cycle progression to the four-cell stage (no cytotstatic arrest) or to the three-cell stage (cytotstatic arrest). Representative examples of arrested and not arrested embryos are shown in (A). (B) Summary of the results obtained. Each bar corresponds to four separate experiments in which we analysed a total of at least 35 embryos. Data represent average ± standard deviation from these experiments. (C) Western blot analyses of proteins in cell extracts (first three rows) or co-immunoprecipitating with HA-Rsk2 (three bottom rows). Hek293 cells were transfected with empty vectors or myc-Emi1, Emi1₂₃₆₋₃₁₃ and HA-p90^{Rsk2} either alone or in combination. A 20 μg portion of total extracts was loaded in each lane (first three rows) and 500 μg of total proteins was immunoprecipitated for each sample (last three rows).

Interaction between p90^{Rsk2} and Emi1 is required during oocyte maturation

Since GST-Emi1₂₃₆₋₃₁₃ acts as an inhibitor of the functional interaction between p90^{Rsk2} and Emi1 *in vivo*, we set out to

determine the role of this interaction during oocyte maturation, when cytotstatic activity is physiologically established. Oocytes at the germinal vesicle stage were allowed to undergo germinal vesicle breakdown (GVBD) in culture. After GVBD, oocytes were microinjected with either GST as control or GST-Emi1₂₃₆₋₃₁₃ and incubated for additional 12–14 h to complete maturation. Control or GST-injected oocytes reached the metaphase II arrest typical of ovulated oocytes; the chromosomes were aligned at the equator of the meiotic spindle, which was located at the periphery of the cell (Figure 7A, Ba, b and C). On the contrary, microinjection of GST-Emi1₂₃₆₋₃₁₃ caused abnormal maturation, with 30% of the oocytes extruding a second polar body (Figure 7A and C). Interestingly, in some oocytes, the polar body was much larger than normal (Figure 7A), indicating a defect in asymmetric division similar to that observed in *mos*^{-/-} oocytes (Choi *et al*, 1996; Verlhac *et al*, 2000b). Immunofluorescence analysis of GST-Emi1₂₃₆₋₃₁₃-injected oocytes revealed either abnormal spindles with misaligned chromosomes (Figure 7Bc and d) or the absence of a meiotic spindle and completion of anaphase (Figure 7Be). On the other hand, the GST-Emi1₂₃₆₋₃₁₃ST/AA mutant did not cause alterations of meiotic progression (Figure 7Bf and C), suggesting that it was unable to interfere with Emi1 phosphorylation *in vivo*. These results highlight the lack of a normal metaphase II arrest in oocytes injected with wild-type GST-Emi1₂₃₆₋₃₁₃ and indicate that the functional interaction between Emi1 and p90^{Rsk2} is required for normal meiotic progression.

RNAi of Emi1 interferes with mouse oocyte maturation

To test whether the morphological defects observed in maturing oocytes injected with the GST-Emi1₂₃₆₋₃₁₃ were due to alterations of Emi1 function, we set out to interfere with RNA expression in oocytes by RNAi. It was shown that microinjection of 300–500 bp double-stranded RNA (dsRNA) sequences in maturing mouse oocytes is capable of specifically depleting endogenous mRNAs (Svoboda *et al*, 2000). Thus, we *in vitro* synthesized dsRNAs comprising the 3' region of mouse Emi1 mRNA, or of GFP mRNA as control, and microinjected them into GV oocytes. Oocytes were allowed to mature and Emi1 mRNA levels were measured in GFPdsRNA- or Emi1dsRNA-injected oocytes. As shown in Figure 8A, Emi1 mRNA was readily detected in GFPdsRNA-injected oocytes, whereas it was absent in Emi1dsRNA-injected oocytes. Interestingly, depletion of Emi1 caused similar morphological defects as those observed by injection of GST-Emi1₂₃₆₋₃₁₃. We observed that 30% of Emi1dsRNA-injected oocytes extruded a second polar body (Figure 8C and Bb) or a much larger polar body indicating the lack of asymmetric division as reported for *mos*^{-/-} oocytes (Figure 8Bc and d), whereas 60% of the oocytes displayed abnormal spindles with scattered chromosomes or decondensed chromatin (Figure 8Bc, d and C). By contrast, GFPdsRNA-injected oocytes underwent normal maturation and arrested with a metaphase II spindle (Figure 8Ba and C). These results indicate that oocyte maturation was severely impaired by selectively interfering with Emi1 expression in mouse oocytes.

Discussion

The CSF activity present in vertebrate oocytes requires activation of the *Mos*/MAPK/p90^{Rsk2} pathway during oocyte

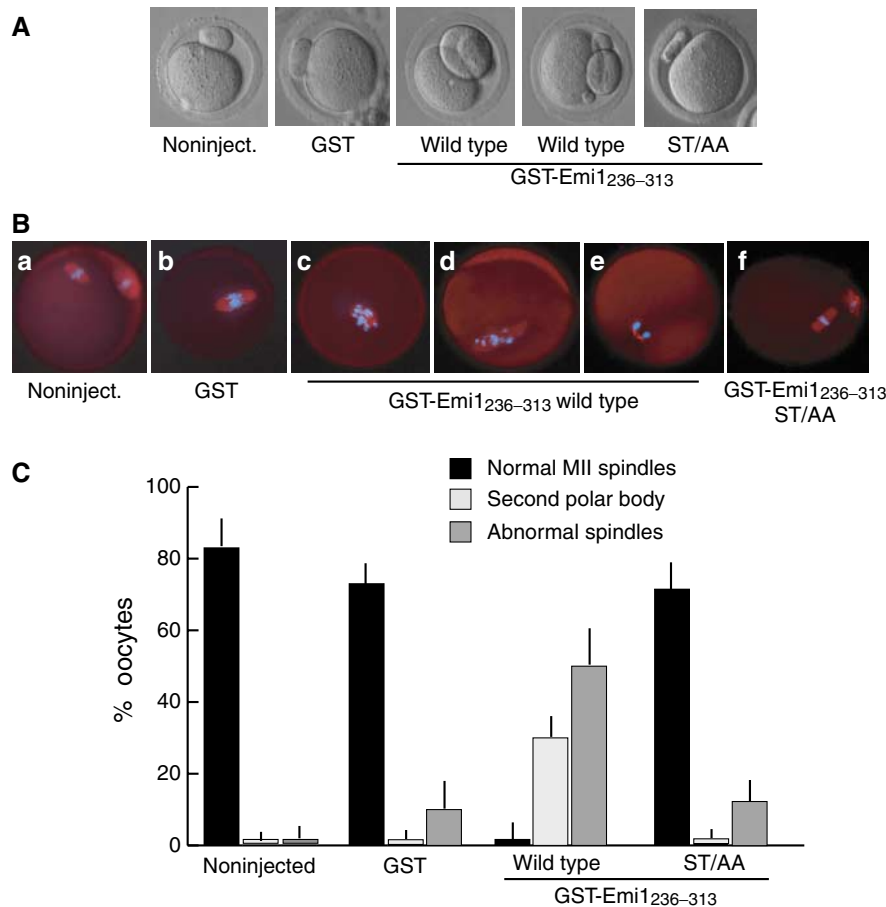


Figure 7 GST-Emi1²³⁶⁻³¹³ interferes with oocyte maturation and cytotstatic arrest. Mouse oocytes were collected from PMSG-primed mice and let undergo GVBD in culture. Immediately after GVBD, oocytes were microinjected with 5–8 pl of GST (1 mg/ml) or either wild-type or ST/AA mutant GST-Emi1²³⁶⁻³¹³ (1 mg/ml) and cultured for additional 14–16 h. **(A)** Representative phase contrast images of control or microinjected oocytes, showing that GST-Emi1²³⁶⁻³¹³ induces either abnormal maturation or extrusion of the second polar body. **(B)** Immunofluorescence analysis of the meiotic spindle in microinjected oocytes. Oocytes were fixed and stained with anti-tubulin antibody and Hoechst dye for DNA. Control or GST-injected oocytes displayed a metaphase plate with a normal spindle. GST-Emi1²³⁶⁻³¹³-injected oocytes displayed either abnormal spindle (c, d) or decondensed chromatin in anaphase position (e), whereas GST-Emi1^{ST/AA}₂₃₆₋₃₁₃-injected oocytes displayed a normal maturation with an MII spindle (f). **(C)** Summary of the results obtained is shown. Abnormal spindles are considered oocytes as in (Bc) or (Bd). Each bar corresponds to average \pm standard deviation from four separate experiments in which we analysed a total of at least 35 oocytes.

maturation and APC^{Cdc20} inhibitory regulators, such as Emi1 and Mad1, to be maintained once it is established (Reimann and Jackson, 2002; Tunquist *et al*, 2003). However, the connection between the factors required for the establishment and the maintenance of CSF is still unclear. Herein, we report that p90^{Rsk2}, the effector of the Mos pathway, directly interacts with and phosphorylates Emi1, an essential inhibitor of APC at metaphase (Reimann and Jackson, 2002). Moreover, we show that functional interaction between p90^{Rsk2} and Emi1 potentiates the ability of Emi1 to bind to Cdc20 and to induce cytotstatic arrest both in mouse blastomeres and in transfected cells. Thus, our results establish a first direct connection between proteins involved in the establishment and in the maintenance of cytotstatic activity in vertebrate eggs.

In vitro binding experiments, supported also by co-immunoprecipitation experiments, indicate that phosphorylation of the C-terminal region of Emi1 by p90^{Rsk2} potentiates four-fold its ability to bind Cdc20. Notably, Reimann and Jackson (2002) showed that cytotstatic arrest was released when recombinant Cdc20 was added to *Xenopus* egg extracts at

3 μ M concentration, but not at 1 μ M concentration, and that it was prevented by addition of equimolar amounts of Emi1. Their results indicate that CSF arrest is maintained by a strict balance between Emi1 and Cdc20 concentrations, and that changing this balance by as little as three-fold triggers metaphase-to-anaphase transition. Thus, we propose that one of the functions of the Mos pathway during oocyte maturation is to reinforce the cytotstatic activity of Emi1 through phosphorylation by p90^{Rsk2} (Figure 9). Our conclusion is supported by the observations that coinjection of Emi1 with p90^{Rsk2} in mouse blastomeres exerts a stronger cytotstatic effect than either protein alone. In addition, substitution of the evolutionarily conserved ser246/thr251 residues, which are phosphorylated by p90^{Rsk2}, suppresses the cooperation between the two proteins *in vivo* and the ability to stabilize the Cdc20/Emi1 interaction *in vitro*. Our results also show that injection of GST-Emi1²³⁶⁻³¹³, which interacts with p90^{Rsk2} and is efficiently phosphorylated by the kinase but is unable to bind Cdc20 efficiently, blocks the cytotstatic activity of constitutively active p90^{Rsk2} in two-cell embryos and it interferes with meiosis II progression and cytotstatic arrest in maturing

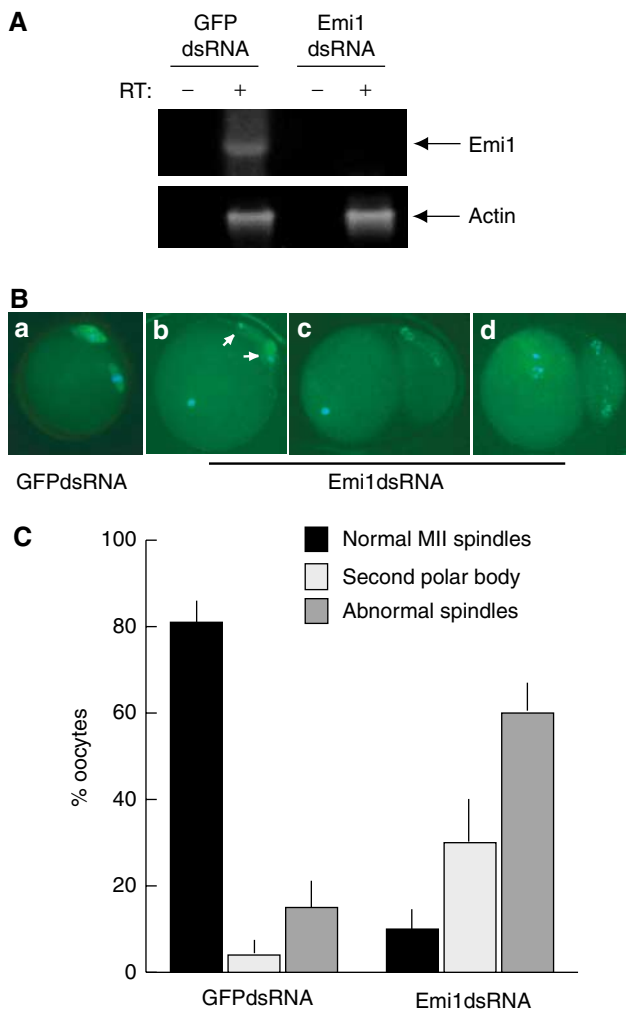


Figure 8 Emi1 RNAi interferes with oocyte maturation and cytostatic arrest. Mouse oocytes were collected from PMSG-primed mice at the GV stage and microinjected with 5–8 pl of either GFPdsRNA (0.5 mg/ml) or Emi1dsRNA (0.5 mg/ml) and cultured for additional 16–20 h to allow maturation. At the end of the incubation, oocytes were scored for the second polar body emission and either frozen in Trizol for RT-PCR analysis or fixed in 4% paraformaldehyde for immunofluorescence analysis. (A) RT-PCR analysis of Emi1 and actin RNA expression in oocytes microinjected with either GFPdsRNA or Emi1dsRNA. A pool of 15 oocytes was used for each sample. Oligonucleotides amplifying a 543 bp sequence in the 5' region of mouse Emi1 (see Materials and methods, oligos for Emi1_{1–181}) were used. (B) Immunofluorescence analysis of the meiotic spindle in microinjected oocytes. Oocytes were fixed and stained with a fluorescein-conjugated anti-tubulin antibody (green) and Hoechst dye for DNA (blue). GFPdsRNA-injected oocytes displayed a metaphase plate with a normal spindle (a). Emi1dsRNA-injected oocytes displayed either decondensed chromatin (b, c) and two polar bodies (white arrows in (b)) or scattered chromosomes and abnormal spindle (d) and they often extruded a larger polar body (c, d). (C) A summary of the results obtained is shown; the score was performed using the criteria described in Figure 7. Each bar corresponds to average \pm standard deviation from four separate experiments in which we analysed a total of at least 40 oocytes.

mouse oocytes. Since similar defects were observed when Emi1 expression was impaired by RNAi, it seems that the effects are due to alterations of Emi1 function. These results suggest that GST-Emi1_{236–313} competes for the interaction of

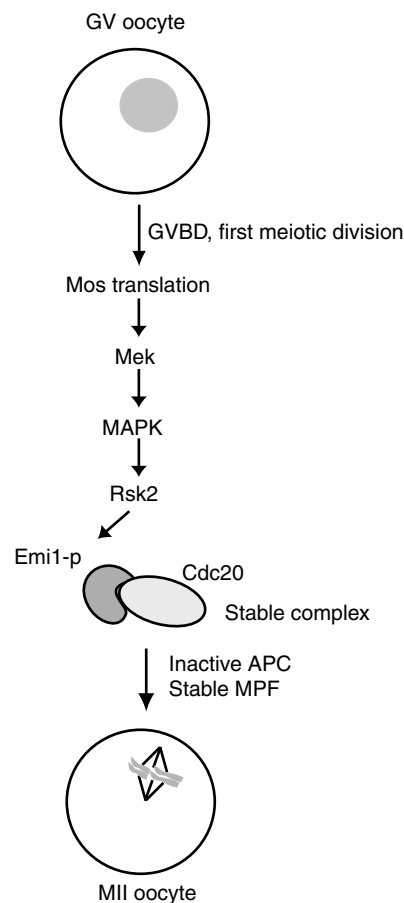


Figure 9 Hypothetical model of CSF establishment in mouse oocytes. Schematic representation of the model hypothesized on the basis of data in the literature and the new findings of this work. See description in the text for a detailed explanation.

p90^{Rsk2} with endogenous Emi1 and that this interaction is necessary for the establishment of cytotstatic arrest.

The morphological examination of oocytes matured in the presence of GST-Emi1_{236–313}, or where Emi1 expression was decreased by RNAi, revealed that they closely resemble *mos*^{-/-} oocytes (Choi *et al*, 1996; Verlhac *et al*, 2000a, b), with some oocytes extruding a second polar body and others with abnormal spindles and misaligned chromosomes or abnormal cytokinesis. Beside stabilization of MPF activity through inhibition of the APC^{Cdc20}, the Mos/MAPK/p90^{Rsk} pathway is also important for stabilization of the spindle through post-translational modifications of microtubule-associated proteins like MISS and DOC1R (Verlhac *et al*, 1996; Lefebvre *et al*, 2002; Terret *et al*, 2003). The spindle abnormalities in oocytes injected with GST-Emi1_{236–313} or Emi1dsRNA suggest that the functional interaction between Emi1 and p90^{Rsk2} contributes also to this aspect of meiosis II. Interestingly, it has been shown that MISS protein is stable only in metaphase II-arrested oocytes, once the cytotstatic activity has been established. Since the spindle defects observed after *in vivo* depletion of MISS (Lefebvre *et al*, 2002) closely resemble those obtained by interference with Emi1, it would be interesting to determine whether MISS is a substrate for the APC^{Cdc20} and whether Emi1 prevents its degradation in meiosis.

The only proteins shown to be required for maintenance of the cytostatic arrest in eggs are the APC^{Cdc20} inhibitors Emi1 and Mad1 (Reimann and Jackson, 2002; Tunquist *et al*, 2003). In both cases, the crucial experiments employed an immunodepletion approach from *Xenopus* egg extracts, which are typically arrested at metaphase with high MPF and MAPK activity. Depletion of either Mad1 or Emi1 led to activation of the APC, cyclin B degradation and mitotic exit. Since addition of either recombinant Mad1 or Emi1 was sufficient to block these events, in both works it was concluded that these proteins were necessary and sufficient to maintain the CSF arrest of *Xenopus* eggs. One possibility to reconcile this apparent paradox is that Mad1 and Emi1 associate in a complex and depletion of either one causes mitotic exit because of depletion of the inhibitory complex. However, it was not checked whether or not Emi1 co-immunoprecipitates with Mad1 in the immunodepletion experiments (Reimann and Jackson, 2002; Tunquist *et al*, 2003). Because of the limiting volume of mouse oocytes, such approaches cannot be applied to our system. Nevertheless, our *in vivo* competition and/or depletion approaches suggest that Emi1 participates in cytostatic arrest also in mouse oocytes.

Our observations suggest that the metaphase-to-anaphase transition in vertebrate eggs is controlled by a relay of phosphorylation events that fine-tune the reciprocal affinity of regulatory components of the APC. In our model, p90^{Rsk2} is activated by the Mos pathway during oocyte maturation and phosphorylates Emi1, increasing its affinity for Cdc20 and preventing activation of the APC (Figure 9). After metaphase is reached, the Mos pathway becomes dispensable (Tunquist and Maller, 2003), possibly because phosphorylated Emi1 is stable in the oocyte environment. This might be due to the absence of a counteracting phosphatase or to inaccessibility of Emi1 when complexed to Cdc20. In agreement with this second hypothesis, it was noted that Emi1 has a stronger affinity for the APC activator Cdh1 than for Cdc20, and it is rapidly degraded when Cdh1 disappears (Reimann *et al*, 2001b; Hsu *et al*, 2002). Since only Cdc20 is present in oocytes (Lorca *et al*, 1998), it is possible that phosphorylation by p90^{Rsk2} prolongs Emi1 life in meiosis through the stabilization of the normally loose interaction with Cdc20. At fertilization, activation of CaMKII triggers activation of APC^{Cdc20} and degradation of cyclin B (Lorca *et al*, 1993). It remains to be established whether CaMKII acts directly through phosphorylation of Emi1 or Cdc20 and whether phosphorylation causes dissociation of the complex. In this regard, our preliminary data suggest that at least Emi1 is not a direct *in vitro* substrate for CamKII (MP Paronetto and C Sette, unpublished observation).

In conclusion, our results indicate that p90^{Rsk2} functionally interacts with Emi1 in the establishment of CSF activity in mouse eggs. Our model offers a reconciling view on the connection between two components of CSF that were previously considered independent, leaving puzzling doubts on the relation between the establishment and the maintenance of CSF arrest (Duesbery and Vande Woude, 2002). Moreover, since activation of the MAPK pathway is required also in the response to the spindle- and DNA-damage checkpoints in mitotic cells (Chung and Chen, 2003; Panta *et al*, 2004), our results may provide a direct link between this pathway and cell cycle arrest through the inhibition of the APC.

Materials and methods

Plasmid construction

Mouse Emi1 was amplified by PCR using a 13 dpc embryo cDNA library and oligonucleotides 5'-AGGAATTCATGAAGTGTTTAATTGCAACCCTG-3' (forward) and 5'-GGTCCGACTACAATCTTTGTAAGTTCTTTTAC-3' (reverse) and subcloned into the *EcoRI* and *Sall* sites of either pCDNA3-myc or pGEX4T1 expression vectors for myc- or GST-tagged Emi1, respectively. Oligonucleotides were derived from the mouse Emi1 homologue deposited in the NCBI database (Fbxo5: NM_025995). Additional oligonucleotides used were as follows: 5'-GGTCCGACTCATAGGTGCTCCAGGCCAT-3' (reverse) for GST-Emi1₁₋₁₈₁, 5'-AGGAATTCATGCAGCGAGTCATTGAAGC-3' (forward) for Emi1₂₃₆₋₃₈₃, 5'-GGTCCGACTCAGGCTT TGAGGCTTTCGTTG-3' (reverse) for Emi1₂₃₆₋₃₁₃. Point mutations in Emi1 were introduced by using mutagenic oligonucleotides and PCR amplification. *Pfu* polymerase (Stratagene) was used for all amplifications and constructs sequences were verified by direct sequencing. The vector pMT2-HAp90^{Rsk2} was a generous gift of Dr Mortin Frodin.

Expression and purification of GST fusion proteins. Plasmids (pGEX-) containing GST fusion proteins were transformed into the *Escherichia coli* BL21 strain, and grown at 30°C in LB medium to an OD₆₀₀ = 0.6 before induction with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG, Sigma-Aldrich) for 3 h. GST fusion proteins were purified from bacterial lysates on glutathione-agarose (Sigma-Aldrich) as previously described (Sette *et al*, 1998) and analysed by SDS-PAGE and Coomassie blue staining to test purity and integrity.

Cell culture and transfections. Hek293 cells were maintained in Dulbecco's medium supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) in 90 mm dishes. Subconfluent monolayers were processed for CaPO₄ transfection with 1–10 μg of the appropriate plasmids or by Fugene (Stratagene) with 0.2–2 μg of the appropriate plasmids as previously described (Sette *et al*, 2002). At 24–48 h after transfection, cells were harvested in lysis buffer (50 mM Hepes, pH 7.5, 75 mM NaCl, 10 mM β-glycerophosphate, 2 mM EGTA, 15 mM MgCl₂, 0.1 mM sodium orthovanadate, 1 mM DTT, 0.5% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich)) and incubated for 10 min on ice. Lysates were centrifuged for 10 min at 10 000 g at 4°C and used for further analysis. Protein concentration was determined using a protein assay kit (Bio-Rad) following the manufacturer's instructions.

FACS sorting. Transfected cells were separated based on size (forward scatter) and green fluorescence (GFP-positive) using a FACS Vantage cell sorter (Beckton and Dickinson). Purity of GFP-positive and -negative populations was >98%. Sorted cells were used for Western blot analysis as described below.

Pull-down assays. Cell extracts (500 μg of total proteins) were added to 2 μg of GST fusion protein adsorbed on glutathione-agarose (Sigma-Aldrich) in 250 μl (final volume) of lysis buffer supplemented with 0.05% bovine serum albumin (BSA). After incubation for 90 min at 4°C under constant shaking, beads were washed three times with lysis buffer without Triton X-100, and adsorbed proteins were eluted in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (wt/vol) SDS, 0.7 M 2-mercaptoethanol and 0.0025% (wt/vol) bromophenol blue) and resolved on a 10% SDS-PAGE for subsequent Western blot analysis.

Immunoprecipitation assay. Cell extracts (500 μg of total proteins) were incubated with 1 μg of anti-myc antibody for 2 h at 4°C under constant shaking. Protein A-Sepharose or protein G-Sepharose (Sigma-Aldrich) was preadsorbed with 0.05% BSA before incubation with the immunocomplexes for an additional hour. Hence, beads were washed three times with lysis buffer and adsorbed proteins were eluted in SDS sample buffer for Western blot analysis.

Kinase assays. For p90^{Rsk2} assays, 1 μg of each GST-Emi1 fusion protein was incubated at 30°C for 20 min with the purified active form of the kinase (5 U, Upstate Biotechnology) in reaction buffer: 50 mM Hepes, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 0.5 mM NaVO₄, 50 μM ATP and 5 μCi of

³²P- γ -ATP. In some experiments, GST fusion proteins were phosphorylated while still bound to the GSH-agarose beads and at the end of the incubation the kinases were washed by rinsing three times with an excess of kinase buffer (without label) before using the proteins for pull-down assays. H1 kinase assays were performed on cell extracts of GFP-positive Hek293 cells as previously described (Bhatt and Ferrell, 1999).

Western blot analysis. Cell extracts or immunoprecipitated proteins were diluted in SDS sample buffer as described above and boiled for 5 min. For oocyte extracts, 300 metaphase II oocytes/sample were collected and immediately frozen in sample buffer. After thawing, oocytes were sonicated and boiled before loading. Proteins were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore) using a semidry blotting apparatus (Bio-Rad). The membranes were saturated with 5% nonfat dry milk in PBS containing 0.1% Tween 20 for 1 h at room temperature, and incubated with the following primary antibodies (1:1000 dilution) overnight at 4°C: mouse anti-HA (for HA-p90^{Rsk2}, from BabCO Berkeley antibody company); rabbit anti-actin (Sigma-Aldrich); mouse anti-Myc (for myc-Emi1); rabbit anti-Emi1 (Gentaur); goat anti-p90^{Rsk2}, rabbit anti-Cdc20, rabbit anti-cyclin A2, mouse anti-cyclin-B1. Primary antibodies were all from SantaCruz Biotechnology, unless specified otherwise. Secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Amersham) were incubated with the membranes for 1 h at room temperature at a 1:10 000 dilution in PBS containing 0.1% Tween 20. Immunostained bands were detected by chemiluminescent method (SantaCruz Biotechnology).

Immunofluorescence analysis. Oocytes were processed for immunofluorescence analysis using anti-tubulin antibody (1:100, Sigma-Aldrich) or anti-Emi1 antibody (1:200, Gentaur) or anti-p90^{Rsk2} antibody (1:200, SantaCruz Biotechnology) as previously documented (Sette *et al*, 2002).

Oocyte collection, microinjection and in vitro culture

Two-cell embryos and GV oocytes were collected from hormonally primed 6- to 7-week-old CD1 female mice (Charles River Italia) and cultured in M16 medium under mineral oil as previously described (Hogan *et al*, 1994). Oocytes were allowed to undergo GVBD by incubation in the absence of an exogenous cAMP source and used for microinjection either immediately (for dsRNAi) or after GVBD (approximately 2 h after collection). Before injection, oocytes and embryos were washed in M2 medium and then transferred to 50 μ l drops of the same medium under mineral oil. Microinjection manipulations were performed as previously described (Sette *et al*, 1998). Briefly, into the cytoplasm of one blastomere of a two-cell

embryo we injected 2–5 μ l of a purified p90^{Rsk2} (1–5 U, Upstate Biotechnology) together with either GST or GST-Emi1 diluted to a protein concentration of 1 mg/ml in injection buffer (20 mM HEPES, pH 7.4, 120 mM KCl, 100 μ M EGTA, 10 mM β -glycerophosphate, 1 mM DTT, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin). Microinjections were performed using an Olympus inverted microscope (Olympus) equipped with Hoffman modulation contrast optics (Modulation optics Inc., Greenvale, NY) and two Leitz mechanical micromanipulators (Leica AG, Heerbrugg, Switzerland). After microinjections, embryos or oocytes were returned to M16 medium drops and cultured at 37°C under a humidified atmosphere of 5% CO₂ in air. At 12–14 h after injection, cells were scored for mitotic or meiotic divisions or processed for immunofluorescence analysis.

dsRNA preparation

To generate templates for dsRNA synthesis, we employed forward and reverse oligonucleotides containing at the 5' end a T7 promoter sequence. For Emi1 amplification, the following primers were designed: forward 5'-GTAATACGACTCACTACTATAGGGCATGCAGC GAGTCA-3'; reverse 5'-GTAATACGACTCACTACTATAGGGCTCA CAATCTTTGT. These oligonucleotides amplify a region of 447 bp from base 945 to 1392 at the 3' end of mouse Emi1 (AK011820). For GFP amplification, the following primers were designed: forward 5'-GTAATACGACTCACTACTATAGGGCATGATAAAGGAG-3'; reverse 5'-GTAATACGACTCACTACTATAGGGCTCAATGCATTAGTTC-3'. These oligonucleotides amplify a region of 600 bp of the GFP sequence. pCDNA3-mycEmi1 and pCMV5-GFP expression vectors were used as templates for PCR amplification. Amplified bands were gel-purified and used as templates (1 μ g) for *in vitro* RNA transcription in order to obtain sense and antisense RNA sequences as previously described (Svoboda *et al*, 2000). RNA was extracted and precipitated by standard procedures and dissolved in RNase-free H₂O. Equimolar amounts of sense and antisense RNA were annealed in DEPC-water supplemented with 1 U/ μ l of RNasin (Invitrogen) and 4 μ g of RNA was boiled for 1 min and allowed to cool down at room temperature before phenol/chloroform extraction and ethanol precipitation. dsRNA was resuspended in H₂O, assayed by agarose electrophoresis and stored at –80°C prior to use.

Acknowledgements

We thank Drs Francesco Cenci and Federica Capolunghi for their help with FACS analysis, Drs Manuela Pellegrini and Susanna Dolci for critically reading the manuscript and Dr Mortin Frodin for the gift of the pMT2-HA-p90^{Rsk2} vector. This work was supported by MIUR Cofin 2002 and 2003.

References

- Bhatt RR, Ferrell Jr JE (1999) The protein kinase p90 rsk as an essential mediator of cytotstatic factor activity. *Science* **286**: 1362–1365
- Bhatt RR, Ferrell Jr JE (2000) Cloning and characterization of *Xenopus* Rsk2, the predominant p90 Rsk isozyme in oocytes and eggs. *J Biol Chem* **275**: 32983–32990
- Choi T, Fukasawa K, Zhou R, Tessarollo L, Borrer K, Resau J, Vande Woude GF (1996) The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. *Proc Natl Acad Sci USA* **93**: 7032–7035
- Chung E, Chen RH (2003) Phosphorylation of Cdc20 is required for its inhibition by the spindle checkpoint. *Nat Cell Biol* **5**: 748–753
- Colledge WH, Carlton MB, Udy GB, Evans MJ (1994) Disruption of c-mos causes parthenogenetic development of unfertilised mouse eggs. *Nature* **370**: 65–68
- Duesbery NS, Vande Woude GF (2002) Developmental biology: an arresting activity. *Nature* **416**: 804–805
- Dupr e A, Jessus C, Ozon R, Haccard O (2002) Mos is not required for the initiation of meiotic maturation in *Xenopus* oocytes. *EMBO J* **21**: 4026–4036
- Gross SD, Schwab MS, Lewellyn AL, Maller JL (1999) Induction of metaphase arrest in cleaving *Xenopus* embryos by the protein kinase p90Rsk. *Science* **286**: 1365–1367
- Gross SD, Schwab MS, Taieb FE, Lewellyn AL, Qian YW, Maller JL (2000) The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by p90(Rsk). *Curr Biol* **10**: 430–438
- Haccard O, Sarcevic B, Lewellyn A, Hartley R, Roy L, Izumi T, Erikson E, Maller JL (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, Aizaway S (1994) Parthenogenetic activation of oocytes in c-mos-deficient mice. *Nature* **370**: 68–71
- Hogan B, Beddington R, Costantini F, Lacy E (1994) *Manipulating the Mouse Embryo*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Hsu JY, Reimann JD, Sorensen CS, Lukas J, Jackson PK (2002) E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). *Nat Cell Biol* **4**: 358–366
- Kalab P, Kubiak JZ, Verlhac MH, Colledge WH, 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
- Kosako H, Gotoh Y, Nishida E (1994) Mitogen-activated protein kinase kinase is required for the mos-induced metaphase arrest. *J Biol Chem* **269**: 28354–28358

- Lefebvre C, Terret ME, Djiane A, Rassinier P, Maro B, Verlhac MH (2002) Meiotic spindle stability depends on MAPK-interacting and spindle-stabilizing protein (MISS), a new MAPK substrate. *J Cell Biol* **157**: 603–613
- Lorca T, Castro A, Martinez AM, Vigneron S, Morin N, Sigrist S, Lehner C, Doree M, Labbe JC (1998) Fizzy is required for activation of the APC/cyclosome in *Xenopus* egg extracts. *EMBO J* **17**: 3565–3575
- Lorca T, Cruzalegui FH, Fesquet D, Cavadore JC, Mery J, Means A, Doree M (1993) Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366**: 270–273
- Margottin-Goguet F, Hsu JY, Koktev A, Hsieh HM, Reimann JDR, Jackson PK (2003) Prophase destruction of Emi1 by the SCF(betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. *Dev Cell* **4**: 813–826
- Markoulaki S, Matson S, Abbott AL, Ducibella T (2003) Oscillatory CaMKII activity in mouse egg activation. *Dev Biol* **258**: 464–474
- Masui Y (2001) From oocyte maturation to the *in vitro* cell cycle: the history of discoveries of maturation-promoting factor (MPF) and cytostatic factor (CSF). *Differentiation* **69**: 1–17
- Masui Y, Markert CL (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J Exp Zool* **177**: 129–145
- Panta GR, Kaur S, Cavin LG, Cortes ML, Mercurio F, Lothstein L, Sweatman TW, Israel M, Arsura M (2004) ATM and the catalytic subunit of DNA-dependent protein kinase activate NF-kappaB through a common MEK/extracellular signal-regulated kinase/p90(rsk) signaling pathway in response to distinct forms of DNA damage. *Mol Cell Biol* **24**: 1823–1835
- Reimann JD, Freed E, Hsu JY, Kramer ER, Peters JM, Jackson PK (2001a) Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. *Cell* **105**: 645–655
- Reimann JD, Gardner BE, Margottin-Goguet F, Jackson PK (2001b) Emi1 regulates the anaphase-promoting complex by a different mechanism than Mad2 proteins. *Genes Dev* **15**: 3278–3285
- Reimann JDR, Jackson PK (2002) Emi1 is required for cytostatic factor arrest in vertebrate eggs. *Nature* **416**: 850–854
- Sagata N, Oskarsson M, Copeland T, Brumbaugh J, Vande Woude GF (1988) Function of c-mos proto-oncogene product in meiotic maturation in *Xenopus* oocytes. *Nature* **335**: 519–525
- Schwab MS, Roberts BT, Gross SD, Tunquist BJ, Taieb FE, Lewellin AL, Maller JL (2001) Bub1 is activated by the protein kinase p90(Rsk) during *Xenopus* oocyte maturation. *Curr Biol* **11**: 141–150
- Sette C, Bevilacqua A, Geremia R, Rossi P (1998) Involvement of phospholipase Cgamma1 in mouse egg activation induced by a truncated form of the C-kit tyrosine kinase present in spermatozoa. *J Cell Biol* **142**: 1063–1074
- Sette C, Paronetto MP, Barchi M, Bevilacqua A, Geremia R, Rossi P (2002) Tr-kit-induced resumption of the cell cycle in mouse eggs requires activation of a Src-like kinase. *EMBO J* **21**: 5386–5395
- Svoboda P, Stein P, Hayashi H, Schultz RM (2000) Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development* **127**: 4147–4156
- Terret ME, Lefebvre C, Djiane A, Rassinier P, Moreau J, Maro B, Verlhac MH (2003) DOC1R: a MAP kinase substrate that control microtubule organization of metaphase II mouse oocytes. *Development* **130**: 5169–5177
- Tunquist BJ, Eysers PA, Chen LG, Lewellyn AL, Maller JL (2003) Spindle checkpoint proteins Mad1 and Mad2 are required for cytostatic factor-mediated metaphase arrest. *J Cell Biol* **163**: 1231–1242
- Tunquist BJ, Maller JL (2003) Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes Dev* **17**: 683–710
- Tunquist BJ, Schwab MS, Chen LG, Maller JL (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
- Verlhac MH, Kubiak JZ, Weber M, Geraud G, Colledge WH, Evans MJ, 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
- Verlhac MH, Lefebvre C, Guillaud P, Rassinier P, Maro B (2000b) Asymmetric division in mouse oocytes: with or without Mos. *Curr Biol* **10**: 1303–1306
- Verlhac MH, Lefebvre C, Kubiak JZ, Umbhauer M, Rassinier P, Colledge W, Maro B (2000a) Mos activates MAP kinase in mouse oocytes through two opposite pathways. *EMBO J* **19**: 6065–6074