p90Rsk is not involved in cytostatic factor arrest in mouse oocytes

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Vertebrate oocytes arrest in metaphase of the second meiotic division (MII), where they maintain a high cdc2/cyclin B activity and a stable, bipolar spindle because of cytostatic factor (CSF) activity. The Mos–MAPK pathway is essential for establishing CSF. Indeed, oocytes from the *mos*−/− strain do not arrest in MII and activate without fertilization, as do *Xenopus laevis* oocytes injected with morpholino oligonucleotides directed against Mos. In *Xenopus* oocytes, p90Rsk (ribosomal S6 kinase), a MAPK substrate, is the main mediator of CSF activity. We show here that this is not the case in mouse oocytes. The injection of constitutively active mutant forms of Rsk1 and Rsk2 does not induce a cell cycle arrest in two-cell mouse embryos. Moreover, these two mutant forms do not restore MII arrest after their injection into *mos*−/− oocytes. Eventually, oocytes from the triple Rsk (1, 2, 3) knockout present a normal CSF arrest. We demonstrate that p90Rsk is not involved in the MII arrest of mouse oocytes.

Introduction

Most oocytes from vertebrate species are naturally arrested in metaphase of the second meiotic division (MII), and harbor a stable spindle with sister chromatids aligned on the metaphase plate. Fertilization triggers sister chromatid separation and the extrusion of the second polar body, as well as entry into embryonic development. The activity that keeps oocytes arrested in MII was first identified in *Rana pipiens* eggs and was called cytostatic factor (CSF) by Masui and Markert (1971). This activity, when injected into a two-cell *Xenopus laevis* embryo, induces a cell cycle arrest in the M phase. The precise molecular nature of CSF has not been characterized, but it has been unambiguously shown in *Xenopus* and mouse oocytes that the Mos–MAPK pathway is required for generating CSF activity. Indeed, *mos* knockout oocytes, which do not activate MAPK from the extracellular regulated kinase (ERK) 1/2 family, as well as *Xenopus* oocytes injected with anti-Mos morpholino oligonucleotides, activate parthenogenetically (Colledge et al., 1994; Hashimoto et al., 1994; Verlhac et al., 1996; Dupre et al., 2002). In *Xenopus* eggs, p90Rsk (ribosomal S6 kinase), a well-known, in vivo MAPK substrate, has been involved in generating CSF activity. There are two closely related p90Rsk that are present and active in CSF-arrested *Xenopus* eggs: Rsk1 and Rsk2; Rsk2 is the most abundant isoform (Bhatt and Ferrell, 2000). Constitutively active forms of p90Rsk (1 or 2) induce CSF arrest after injection into a *Xenopus* embryo, and do so independently of the activation of the Mos–MAPK pathway (Bhatt and Ferrell, 1999; Gross et al., 1999). Despite the presence of Rsk1, a Rsk2-depleted extract cannot undergo CSF arrest in response to Mos (Bhatt and Ferrell, 1999). Eventually, *Xenopus* oocytes treated with U0126, a MAPK/ERK kinase (MEK) inhibitor, go to interphase after meiosis resumption. Overexpression of constitutively active p90Rsk into U0126-treated *Xenopus* oocytes rescues a normal MII arrest with a stable, bipolar spindle (Gross et al., 2000). Studies from *Xenopus* eggs, therefore, suggest that p90Rsk is the sole mediator of the Mos–MAPK pathway involved in CSF activity (Bhatt and Ferrell, 1999; Gross et al., 1999, 2000).

In mouse oocytes, p90Rsk was also shown to be downstream of the Mos–MAPK pathway (Kalab et al., 1996). However, *mos* knockout oocytes that do not activate MAPK, but still harbor 50% of the residual activity of p90Rsk, do not develop CSF activity (Kalab et al., 1996). Also, two MAPK substrates are necessary for maintaining spindle stability during the CSF arrest in mouse oocytes: MISS (MAPK interacting and spindle stabilizing) protein (Lefebvre et al., 2002) and DOC1R (deleted in oral cancer one related; Terret et al., 2003a). The requirement of these two substrates suggests that p90Rsk is probably not a unique, downstream target of the Mos–MAPK pathway during mouse oocyte meiotic maturation. For these

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Abbreviations used in this paper: CSF, cytostatic factor; ERK, extracellular regulated kinase; GVBD, germinal vesicle breakdown; MEK, MAPK/ERK kinase; MII, metaphase of the second meiotic division.

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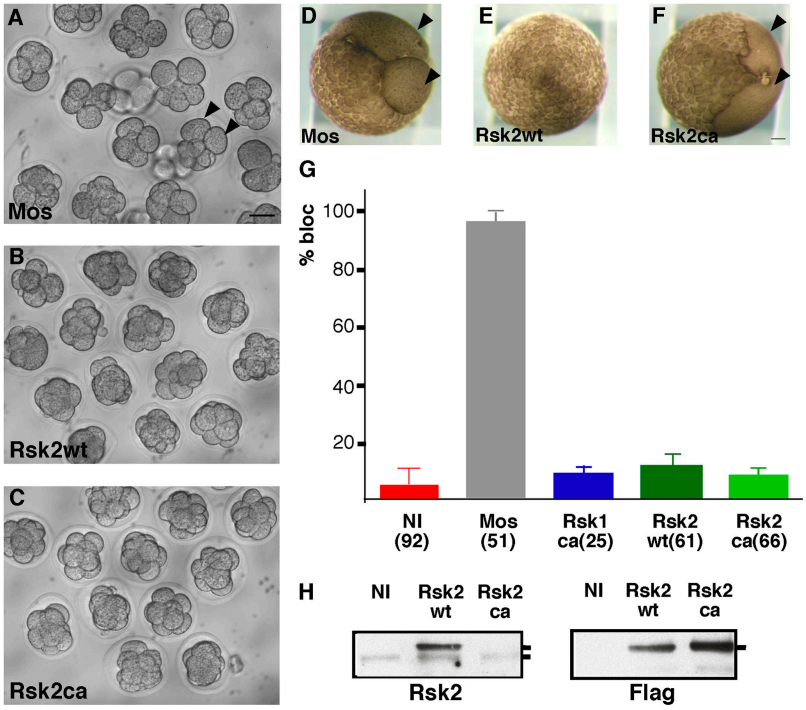

Figure 1. **Constitutively active Rsk1 or 2 does not induce CSF arrest in early mouse embryos, but does so in early *Xenopus* embryos.** (A–C) Phase-contrast image of mouse embryos injected at the two-cell stage into one blastomere and observed 24 h later. (A) Cleavage arrest induced after Mos-RNA injection (black arrowheads). No arrest is observed after the injection of RNA-encoding Rsk2wt or Rsk2ca (B and C, respectively). Bar, 50 μm. (D–F) Phase-contrast image of two-cell *Xenopus* embryos injected into one blastomere with RNA-encoding Mos (D), Rsk2wt (E), and Rsk2ca (F). Embryos were observed 3 h after injection. The black arrowheads point toward blastomeres, which undergo cell cycle arrest after injection. Bar, 100 μm. (G) Percentage of cleavage arrest (%bloc) induced in one blastomere of a late two-cell mouse embryo after an injection of RNA-encoding Mos, Rsk1ca, Rsk2wt, and Rsk2ca. The number in parentheses corresponds to the number of embryos that were analyzed. Two-cell embryos were injected between 48 and 50 h after a human chorionic gonadotropin injection, which corresponds to ∼4 h before the two- to four-cell division. Error bars are the SD from three experiments. (H) Immunoblotting of 30 two-cell mouse embryos that were either noninjected (NI) or injected with Rsk2wt or Rsk2ca RNA into one blastomere and collected 24 h after injection. Left panel is revealed with an anti-Rsk2 antibody. Right panel is revealed with an anti-Flag antibody. The anti-Rsk2 recognizes endogenous Rsk2, but not Rsk2ca.

reasons, we decided to determine whether p90Rsk was necessary and sufficient to establish CSF arrest in mouse oocytes. In this manuscript, we demonstrate that proteins of the p90Rsk family are not involved in the MII arrest in mouse oocytes by using both overexpression of constitutively active Rsk1 and 2 in mouse oocytes and embryos, and analyzing the CSF arrest in oocytes from the Rsk (1, 2, 3) knockout strain.

Results and discussion

Injection of Mos or constitutively active MEK into one blastomere of a late, two-cell mouse embryo induces a cell cycle arrest and can be used as a test for CSF activity (Fig. 1; Verlhac et al., 2000). We injected RNA-encoding, constitutively active p90Rsk into one blastomere of a two-cell mouse embryo (Silverman et al., 2004). Although we obtained a 98% block after the injection of Mos, neither constitutively active Rsk1 nor Rsk2 was able to induce a cell cycle arrest above the background level (Fig. 1, compare A–C with G). This was not caused by a lack of overexpression of the mutant forms of Rsk, because, for example, Rsk2wt and Rsk2ca were overexpressed ∼10 times, compared with endogenous Rsk (Fig. 1 H; the anti-Rsk2 antibody recognizes both wild-type Rsk2 and endogenous Rsk2, but does not recognize the constitutively active Rsk2). The same extract probed with an anti-Flag antibody shows that Rsk2ca overexpression was even higher than Rsk2wt overexpression (Fig. 1 H, right). Furthermore, injection of the same RNA preparation that encodes Rsk1ca or Rsk2ca into one blastomere of a two-cell *Xenopus* embryo induces the expected cell cycle arrest in the injected blastomeres (Fig. 1, compare F with D and E; Silverman et al., 2004). Rsk1 and Rsk2 are abundant in *Xenopus* oocytes and early embryos (Silverman et al., 2004). Interestingly, the amount of expression of the exogenous proteins versus the amount of expression of the endogenous proteins is even greater in the mouse than in *Xenopus* (10 times more than the endogenous proteins in the mouse embryo compared with 10 times less than the endogenous proteins in *Xenopus*; Silverman et al., 2004; unpublished data). Thus, whereas Mos or active MEK is sufficient to induce a cell cycle arrest in mouse embryos, constitutively active Rsk is not.

Oocytes from the *mos*−/− strain are not arrested in MII, but activate spontaneously in the absence of fertilization and extrude a second polar body. To determine whether p90Rsk was able to restore MII arrest in these oocytes, we overexpressed constitutively active Rsk1 or 2. As expected, the injection of RNA-encoding Mos completely rescued the MII arrest in this strain, and an oocyte did not extrude a second polar body (see Fig. 3, B and D). Oocytes from the *mos*−/− strain injected with RNA-encoding Rsk1ca extruded their first and second polar bodies with the same timing as control, noninjected oocytes (Fig. 2 A). Oocytes from this particular experiment, however, overexpressed Rsk1ca, as shown in Fig. 2 B. We observed the same phenotype after an injection of RNA-encoding Rsk2ca (Fig. 3, C and D). Therefore, the injection of RNA-encoding, constitutively active Rsk1 or 2 does not restore MII arrest in *mos*−/− oocytes. The absence of rescue is not caused by a lack of Rsk1ca or Rsk2ca expression (Fig. 2 B; and Fig. 3 E, bottom lanes 2–4), nor is it caused by a lack of activity, because both forms are recognized by an anti-phosphoRsk antibody that is specific for active p90Rsk (Fig. 3 E, top lanes 2–4).

Our data suggested that p90Rsk is not sufficient to induce CSF activity in mouse oocytes. There are four Rsk members in the mouse: Rsk1–4. Though similar in structure to the other Rsk family members, Rsk4 has a function that is distinct from that of Rsk1–3. In the early mouse embryo, Rsk4 expression is

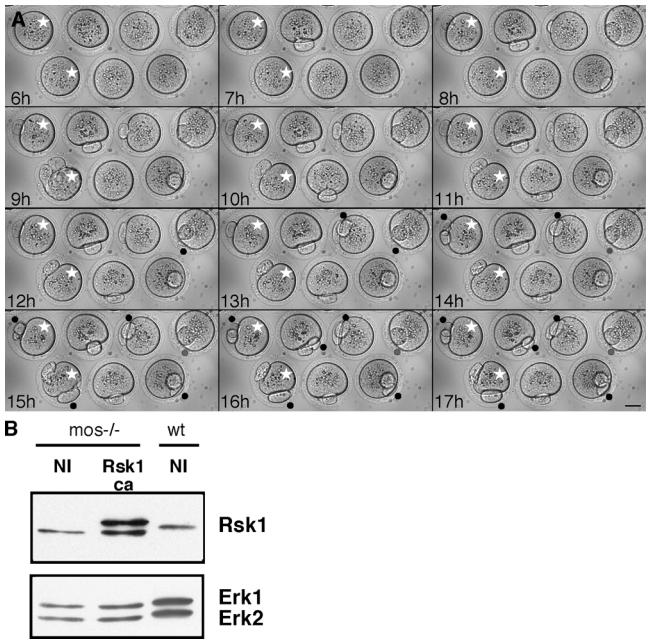

Figure 2. **Constitutively active Rsk1 does not rescue the CSF arrest of oocytes from the *mos*−/− strain.** (A) Time-lapse microscopy of maturing oocytes from the Mos-deficient strain, either noninjected (white stars) or injected with RNA-encoding Rsk1ca. Time points correspond to time after meiosis resumption. Black dots show extrusion of the second polar body. Gray dots correspond to the second polar body moving out of focus. Bar, 50 μm. (B) Immunoblotting of 30 oocytes from the experiment shown in A, either noninjected (NI) or injected with RNA-encoding Rsk1ca. Rsk1ca is overexpressed about five times (top) after injection of the corresponding RNA into oocytes from the *mos*−/− strain. The top panel is revealed with an anti-Rsk1 antibody. The bottom panel is revealed with an anti-ERK antibody and serves as a loading control.

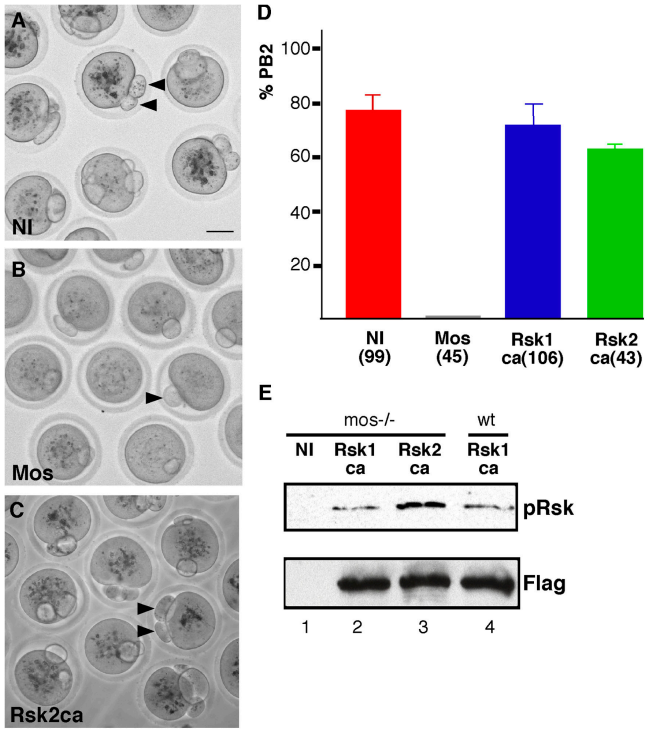

Figure 3. **Constitutively active Rsk1 or 2 does not rescue the CSF arrest of oocytes from the *mos*−/− strain.** (A–C) Phase-contrast images of oocytes from the Mos-deficient strain, either noninjected (A), injected with RNA-encoding Mos (B), or injected with Rsk2ca (C). Arrowheads point toward the polar bodies (two in A and C, and one in B). Bar, 50 μm. (D) Overexpression of Mos, but not of Rsk1ca or Rsk2ca, rescues the MII arrest in oocytes from *mos*−/− mice, as evidenced by spontaneous second polar body extrusion (%PB2). The number in parentheses corresponds to the number of embryos analyzed. Error bars are the SD from three experiments. (E) Immunoblotting of 50 oocytes either noninjected (NI) or injected with RNA-encoding Rsk1ca or Rsk2ca. Rsk1ca and Rsk2ca are overexpressed (bottom) and active (top) after injection of the corresponding RNA into oocytes from the wild-type or *mos*−/− strain. The top panel is revealed with an anti-phosphoRsk antibody. The bottom panel is revealed with an anti-Flag antibody.

inversely correlated to the presence of activated ERK1/2 (Myers et al., 2004). Moreover, in the early *Xenopus* embryo, Rsk4 has an inhibitory role in the transduction of MAPK signaling pathways. This inhibitory role is dependent on a region that is not conserved in Rsk1–3 (Myers et al., 2004). Because activation of the Mos–MAPK pathway is required for the CSF arrest, we can rule out the possibility that Rsk4, a downstream inhibitor of ERK1/2, is involved in mediating this activity. Moreover, we were able to amplify Rsk2 and Rsk4 mRNA from whole ovaries using RT-PCR, but were not able to amplify Rsk4 mRNA from mouse immature oocytes using RT-PCR. However, Rsk2 mRNA was consistently amplified from these oocytes using RT-PCR (unpublished data). This suggests, but does not prove, that the Rsk4 mRNA is either absent or expressed at very low levels in mouse oocytes.

Therefore, in order to determine whether p90Rsk is necessary for MII arrest in mouse oocytes, we checked if oocytes from the recently produced Rsk (1, 2, 3) triple knockout (unpublished data) were able to arrest in MII. The lack of Rsk1–3 expression in these mice is shown in protein extracts from the liver and from mitotic embryonic fibroblasts that were derived from these deficient mice (Fig. 4 D, lanes 3 and 4).

As shown in Fig. 4, immature oocytes from the Rsk (1, 2, 3) knockout strain underwent meiosis resumption, as assessed by germinal vesicle breakdown (GVBD). They extruded their first polar body with a frequency similar to that of control oocytes (97% extrusion of the first polar body compared with 80% in control oocytes). Furthermore, oocytes from this deficient strain were arrested in MII with typical, barrel-shaped spindles (Fig. 4 B). They segregated homologous chromosomes and harbored in MII sister chromatids aligned on the metaphase plate, which is typical of a CSF arrest (Fig. 4 C). Like control oocytes, oocytes from the Rsk (1, 2, 3)–deficient strain remained arrested in MII and never extruded a second polar body like oocytes from the c-*mos* knockout strain did.

Our data demonstrate that p90Rsk is neither necessary nor sufficient to establish a CSF arrest in mouse oocytes (Fig. 4 F). This contradicts the work of Paronetto et al. (2004), which shows that the injection of active Rsk2 protein, and not RNA, into one blastomere of a two-cell embryo blocks the division of the injected blastomere in ∼30% of the cases. However, we assume that this block is, in fact, a delay of the division because Paronetto et al. (2004) did not observe the embryos further than one division of the control blastomere. This delay is likely a result of the trauma caused by the injection of protein into the blastomere.

Our results show that the signaling pathway leading to CSF arrest diverges at the level of p90Rsk between *Xenopus* and mouse. This discrepancy is intriguing. However, even if

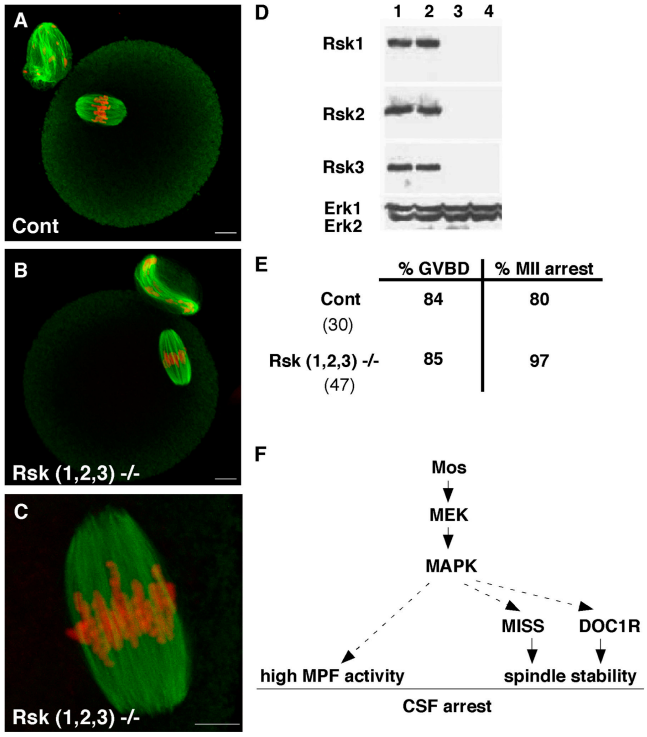

Figure 4. **Oocytes from the Rsk (1, 2, 3)–deficient strain arrest in MII like control oocytes.** (A–C) Immunofluorescent images show the microtubules in green and the chromosomes in red of control wild-type oocytes (A) and oocytes from the Rsk (1, 2, 3)–deficient strain (B and C). Oocytes were observed 19 h after GVBD (~7 h in CSF arrest). (C) An MII spindle of an oocyte from the Rsk (1, 2, 3)–deficient strain is observed under a 63× objective to show the sister chromatids. Bar, 10 μm. (D) Immunoblotting of protein extracts from the mitotic embryonic fibroblasts of control mice (lane 1) or of Rsk (1, 2, 3)–deficient mice (lane 3), and of protein extracts from the liver of control mice (lane 2) or of Rsk (1, 2, 3)–deficient mice (lane 4). Extracts were probed using antibodies specific for Rsk1–3 and ERK1 and 2. (E) Table showing the percentage of oocytes undergoing meiosis resumption (GVBD) and MII arrest in control mice versus that in Rsk (1, 2, and 3)–deficient mice. (F) Scheme of the pathway leading to CSF arrest in the mouse oocyte, which does not go through p90Rsk to maintain a high maturation-promoting factor activity.

the function of the CSF arrest is the same in these two vertebrate species (i.e., preventing parthenogenetic activation), it seems that the mechanisms at play are different. It has been proposed that components of the spindle assembly checkpoint, such as Mad1 and 2, are required for establishing CSF arrest in *Xenopus* (Tunquist et al., 2003). Yet this is not the case in mouse oocytes because the injection of dominant negative mutants of BubR1 or Mad2 still allows the establishment of CSF arrest (Tsurumi et al., 2004). We have recently identified substrates of MAPK that are involved in stabilizing the MII spindle of mouse oocytes, which suggests that the Mos–MAPK pathway has essential targets, other than p90Rsk, on this structure (Lefebvre et al., 2002; Terret et al., 2003a). Mos has other targets, apart from MEK, in mouse oocytes: it inhibits an okadaic acid–sensitive phosphatase, allowing MAPK activation to occur (Verlhac et al., 2000). The observations made in mouse oocytes show that the pathway leading to CSF arrest is not a linear one, as it is in *Xenopus* (Gross et al., 2000). Eventually, in mouse oocytes, cyclin B degradation is partially inhibited during MII arrest and requires an intact spindle to be effective (Kubiak et al., 1993). *Xenopus* oocytes are gigantic cells (1,000 times bigger than a mouse oocyte), whereas their MII spindles are approximately the same size as those in mouse oocytes. The difference between these two species may lie in the way the two types of cells amplify a potential signal coming from a localized region of the cell; i.e., the spindle. Rsk1 and 2 are abundant in *Xenopus* oocytes and embryos (Silverman et al., 2004). We can then hypothesize that in this species, p90Rsk acts as an amplifier of the Mos–MAPK signaling pathway in order to accumulate cyclin B, and hence stabilize maturation-promoting factor activity. However, *mos*−/− oocytes that are injected with Rsk1ca extrude their second polar body with the same timing as control, noninjected oocytes (Fig. 2); this strongly suggests that, in mouse oocytes, cyclin B stability is not regulated by p90Rsk activity. Therefore, our work and the work of Tsurumi et al. (2004) show that many studies performed in the *Xenopus* system are not relevant for mammals.

Materials and methods

Oocyte and embryo collection, culture, and microinjection

Wild-type oocytes and embryos were collected from 11-wk-old OF1 female mice. Oocytes were also collected from the Mos-deficient strain (Colledge et al., 1994) or from the Rsk (1, 2, 3)–deficient strain. Mouse oocytes and embryos were collected, cultured, and microinjected as described previously (Verlhac et al., 2000), and mouse oocytes were processed for immunofluorescence as described in Terret et al. (2003b). *Xenopus* embryos were obtained from females injected with 500 IU of human chorionic gonadotropin (Sigma-Aldrich), were artificially fertilized, and were processed as described previously (Carron et al., 2003). Microinjection of 2.5 ng of capped and purified RNA into one blastomere of a two-cell *Xenopus* embryo was performed in 0.1× MBS (modified Barth's solution) containing 3% Ficoll (Sigma-Aldrich), using a picoinjector (model PLI-100; Medical Systems Corp.). After injection, embryos were maintained in the culture medium for 1 h and were further cultured in 0.1× MBS supplemented with 50 μg/ml gentamicin.

Imaging devices

Phase-contrast images of mouse oocytes and embryos were taken on an inverted microscope (model DMIRBE; Leica; objective lenses 20×/PL fluotar 0.5) equipped with a MicroMax camera (Roper Scientific) and driven with the Metamorph software (Universal Imaging Corp.). The confocal images were taken on an inverted microscope (model TCSSP; Leica; objective lenses 40×/Plan Apo 0.75 and 63×/Plan Apo 1.32). Phase-contrast images of *Xenopus* embryos were taken on a stereomicroscope (model SZX9; Olympus) equipped with a camera (model DXC 950P; Sony).

In vitro transcription of synthetic RNA

The in vitro synthesis and purification of RNA was performed as described in Terret et al. (2003b). The same RNA preparation was injected into mouse oocytes and embryos, as well as into *Xenopus* embryos. The active constructs of rat Rsk1 and mouse Rsk2 are described in Silverman et al. (2004).

Immunoblotting

Immunoblotting of mouse oocytes and embryos was performed as described previously (Verlhac et al., 2000). ERK1 and 2 were recognized using a specific anti-ERK antibody (sc-154; Santa Cruz Biotechnology, Inc.); Rsk1 and 3 were recognized using antibodies sc-231 and sc-13379 (Santa Cruz Biotechnology, Inc.); and Rsk2 from mouse oocytes and embryos was recognized using a specific antibody (sc-1430; Santa Cruz Biotechnology, Inc.). Rsk2 from mitotic embryonic fibroblasts and liver extracts was recognized with the antibody described in Zeniou et al. (2002). Active Rsk was recognized using anti-phospho Rsk (sc-12445; Santa Cruz Biotechnology, Inc.) directed against the PDK1 site present in Rsk1 and 2, which enhances Rsk1 and 2 activity when phosphorylated. The Flag epitope was recognized using an anti-Flag mAb (F3165; Sigma-Aldrich).

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