

Meiotic spindle stability depends on MAPK-interacting and spindle-stabilizing protein (MISS), a new MAPK substrate

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Vertebrate oocytes arrest in the second metaphase of meiosis (metaphase II [MII]) by an activity called cytostatic factor (CSF), with aligned chromosomes and stable spindles. Segregation of chromosomes occurs after fertilization. The *Mos*/.../MAPK (mitogen-activated protein kinases) pathway mediates this MII arrest. Using a two-hybrid screen, we identified a new MAPK partner from a mouse oocyte cDNA library. This protein is unstable during the first meiotic division and accumulates only in MII, where it localizes to the spindle. It is a substrate of the *Mos*/.../MAPK pathway. The depletion of endogenous RNA

coding for this protein by three different means (antisense RNA, double-stranded [ds] RNA, or morpholino oligonucleotides) induces severe spindle defects specific to MII oocytes. Overexpressing the protein from an RNA not targeted by the morpholino rescues spindle destabilization. However, dsRNA has no effect on the first two mitotic divisions. We therefore have discovered a new MAPK substrate involved in maintaining spindle integrity during the CSF arrest of mouse oocytes, called MISS (for MAP kinase-interacting and spindle-stabilizing protein).

Introduction

Meiotic maturation in vertebrate oocytes corresponds to the period including the exit from the G2 block to the arrest in metaphase of the second meiotic division (metaphase II [MII]*). Meiotic maturation starts with the activation of maturation-promoting factor (MPF), which triggers germinal vesicle breakdown (GVBD), chromosome condensation, and formation of the first meiotic spindle. This spindle migrates to the cortex and after extrusion of the first polar body, the second meiotic spindle forms below the cortex, where it remains stable for hours with chromosomes aligned on the metaphase plate.

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*Abbreviations used in this paper: as, antisense; CSF, cytostatic factor; ds, double stranded; ERK2, extracellular regulated kinase 2; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MAPK, mitogen-activated protein kinases; MI, MII, and MIII, metaphase I, II, and III; MISS, MAP kinase-interacting and spindle-stabilizing protein; NLS, nuclear localization signal.

Key words: MISS; MAP kinase; spindle stability; morpholino; mouse

The cascade initiated by *c-mos*, which activates mitogen-activated protein kinases (MAPK) (the *Mos*/.../MAPK pathway), controls many important events of meiotic maturation (for an extensive review see Abrieu et al., 2001). This pathway controls the migration of the germinal vesicle (GV) to the cortex in *Xenopus* oocytes and the migration of the first meiotic spindle to the cortex in mouse oocytes (Choi et al., 1996; Gavin et al., 1999; Verlhac et al., 2000a). It is therefore essential for the establishment of asymmetric divisions during meiosis. This pathway also controls microtubule organization in mouse oocytes (Verlhac et al., 1994, 1996). Moreover, the *Mos*/.../MAPK pathway promotes the cytostatic factor (CSF; Masui and Markert, 1971) arrest of vertebrate oocytes (Haccard et al., 1993; Colledge et al., 1994; Hashimoto et al., 1994; Verlhac et al., 1996). Indeed, vertebrate oocytes are arrested at metaphase of the second meiosis by an egg cytoplasmic substance called CSF. Then fertilization will release the egg from the arrest and allow it to complete meiosis and proceed to mitosis. The CSF arrest of the cell cycle is affected by the *Mos*/.../MAPK cascade, which results in the activation of the ribosomal S6 kinase p90^{sk} (Bhatt and Ferrell, 1999; Gross et al., 1999, 2000). However, the mechanism of the cell cycle arrest in MII has not

been fully clarified. Particularly puzzling is the fact that the cell cycle is not arrested at metaphase I (MI), whereas all the kinases mentioned above have already been activated before MI. So molecules activated only in MII and targets of the Mos/.../MAPK pathway could also be involved in the CSF arrest.

To look for physiological effectors of the Mos/.../MAPK pathway in meiosis, we generated a mouse immature oocyte cDNA library that we screened by two hybrid using MAPK as a bait. We isolated a new protein, MAP kinase-interacting and spindle-stabilizing protein (MISS), which is rich in proline residues and has four potential MAPK phosphorylation sites, a MAPK docking site, a PEST sequence, and a bipartite nuclear localization signal (NLS). Apart from a potential human homologue, we did not find any homologies with other proteins in the databases. In mouse oocytes, MISS is a potential *in vivo* substrate for the Mos/.../MAPK pathway. The endogenous protein accumulates during mouse meiotic maturation and localizes as discrete dots on the MII spindle. Also, a MISS-GFP construct localizes to kinetochore microtubules in MII oocytes. The stability of MISS is regulated during mouse meiotic maturation. It is unstable in MI, where its degradation is microtubule dependent, and becomes stable in MII. MISS is again unstable after parthenogenetic activation and the endogenous protein is no longer detected in early embryos. By microinjecting both antisense (as) RNA, double-stranded (ds) RNA, and morpholino oligonucleotides (Summerton and Weller, 1997) directed against MISS endogenous mRNA, we show that it is involved in maintaining MII spindle integrity. Mouse oocytes microinjected with any of these three different molecules, and not control molecules, display disorganized MII spindles and numerous asters in their cytoplasm. Moreover, the spindle defects induced after microinjection of morpholino oligonucleotides directed against 25 bp upstream of the ATG can be rescued by the overexpression of Myc-MISS from RNA corresponding to the full-length ORF devoid of MISS 5' UTR. The injection of dsRNA against MISS has yet no effect on the first two mitotic divisions, suggesting a specific role for MISS in meiosis. MISS is the first example of a physiological MAPK substrate that is stabilized in MII and specifically regulates MII spindle integrity during the CSF arrest.

Results

Isolation of MISS that binds ERK2

We have prepared a two-hybrid cDNA library from 1,000 mouse immature oocytes. This library was screened using rat extracellular regulated kinase 2 (ERK2, one of the two mammalian MAPK) as a bait. From this screen, six clones were isolated that encoded a partial cDNA of ~500 bp. We confirmed the specificity of the two-hybrid interaction between this partial cDNA product and ERK2 (Fig. 1 A).

A mouse EST showing 95 overlapping base pairs with our partial cDNA enabled us to obtain the full-length ORF from the databases (Fig. 2 A). A full-length clone, identical to ours, has recently been pulled out from an mCAP-enriched cDNA library (Kawai et al., 2001). The sequence of the MISS mRNA and protein is presented in Fig. 2 B. This protein is very rich in proline residues in its NH₂-terminal end and harbors a PEST sequence, a bipartite NLS that con-

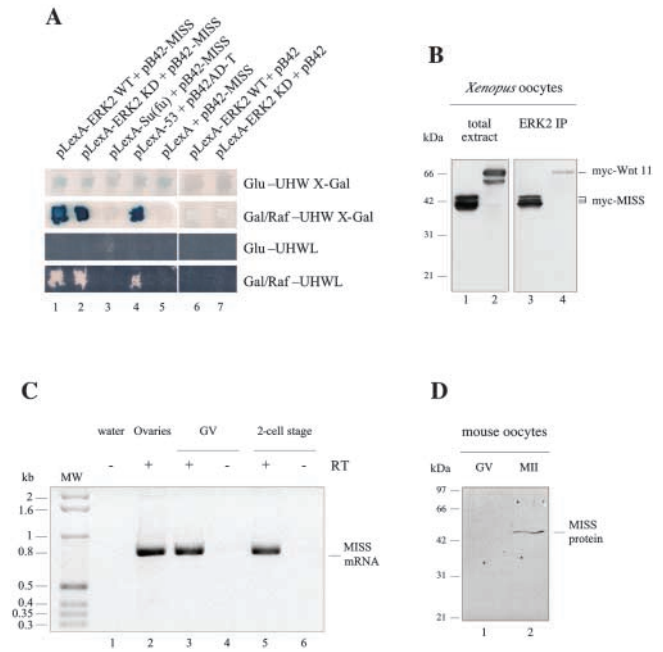


Figure 1. Interaction between MISS and MAPK. (A) Two-hybrid interaction between MISS and MAPK. MISS interacts with ERK2WT and ERK2KD but not with a negative control. The yeast transformed with LexA-ERK2WT, LexA-ERK2KD (Waskiewicz et al., 1997), LexA-53 (positive control; CLONTECH Laboratories, Inc.), LexA-Su(Fu), or LexA alone were mated with yeast transformed respectively with B42-cDNA, B42-AD-T (positive control; CLONTECH Laboratories, Inc.), or empty B42. The diploids obtained were assayed for transactivation of both the β -galactosidase and the LEU2 reporter genes on glucose (Glu)- or galactose (Gal/Raf)-containing mediums. The B42 fusion proteins are under the control of the galactose promoter. The B42-cDNA clearly interacts both with fusions of ERK2WT and ERK2KD at levels identical to the positive control, whereas it does not interact with a negative control (Su[Fu]). (B) MISS coimmunoprecipitates with endogenous p42^{mapk} from *Xenopus* oocyte extracts. Oocyte extracts expressing Myc-Wnt11 (*Xenopus* Wint11; negative control) or Myc-MISS RNA (lanes 1 and 2). Anti-p42^{mapk} immunoprecipitates (lanes 3 and 4) prepared from the oocyte lysates were analyzed by immunoblotting with anti-Myc antibody. This experiment was performed three times. (C) Expression of MISS RNA in immature oocytes and two cell stage embryos. For RT-PCR analysis, total RNA was isolated from mouse ovaries (lane 2), immature oocytes (lanes 3 and 4), and two cell stage embryos (lanes 5 and 6) and treated with or without reverse transcriptase (RT, + or -). Lane 1 corresponds to a PCR control (water). (D) MISS accumulates during meiotic maturation. 2,000 immature (lane 1) or mature (lane 2) oocytes were collected and immunoblotted using an affinity-purified anti-MISS antibody.

tains a MAPK docking site (Tanoue et al., 2000), and potential MAPK phosphorylation sites (Fig. 2 C).

We confirmed the two-hybrid interaction by showing that MISS coimmunoprecipitates with endogenous MAPK from *Xenopus* oocyte extracts (Fig. 1 B).

MISS mRNA is present in immature oocytes but MISS protein accumulates after meiosis resumption and localizes to the MII spindle

To check if the full-length RNA encoding MISS was present in mouse oocytes, we performed RT-PCR using primers specific for the MISS mRNA. By RT-PCR, we could amplify a

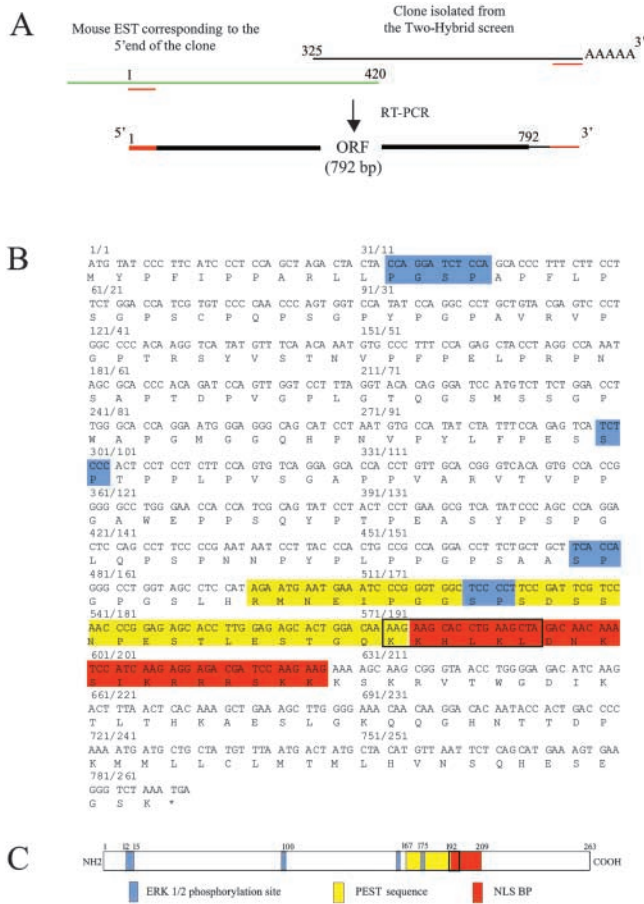


Figure 2. The sequence of MISS. (A) Schematic representation of how the full-length ORF was isolated. (B) Nucleotide and amino acid sequence of MISS ORF (GenBank/EMBL/DDBJ accession no. AK009250). The amino acid sequence is rich in proline in the NH₂-terminal end and contains a PEST sequence, a bipartite NLS and four potential MAPK phosphorylation sites. The MAPK docking site is boxed. (C) Schematic representation of the full-length MISS protein indicating the different domains.

specific band of ~800 bp corresponding to the full-length MISS ORF (Fig. 1 C). As shown in Fig. 1 C, MISS mRNA is expressed in mouse ovaries, mouse immature oocytes (at the GV stage), and two cell stage mouse embryos. However, both by immunoblotting (Fig. 1 D, lane 1) and immunofluorescence (Fig. 3 A) using an affinity-purified anti-MISS peptide antibody, we could not detect the presence of the endogenous protein in immature oocytes. MISS was present in mature oocytes (Fig. 1 D, lane 2). The apparent molecular weight being around 45 kD suggests that MISS undergoes posttranslational modifications. By immunofluorescence, MISS was undetectable in GV oocytes (Fig. 3 A), was hardly detectable in the vicinity of the chromosomes in late MI oocytes (Fig. 3 B), and accumulated strongly and became localized in MII-arrested oocytes (Fig. 3, C and D). Both the cytoplasmic and the MII spindle staining are specific because they are no longer present when the purified antibody is preincubated with the immunogenic peptide (Fig. 3 F). Interestingly, we could not detect any staining in early mouse embryos (Fig. 3 E), suggesting that MISS accumulates specifically in meiosis. A MISS-GFP construct also behaves like the endogenous protein during mei-

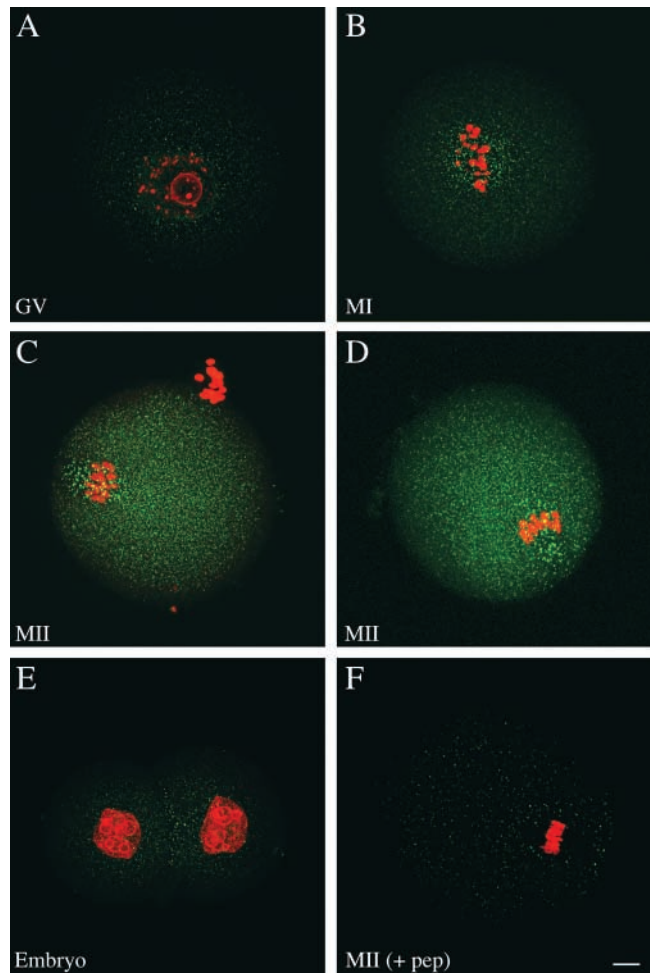


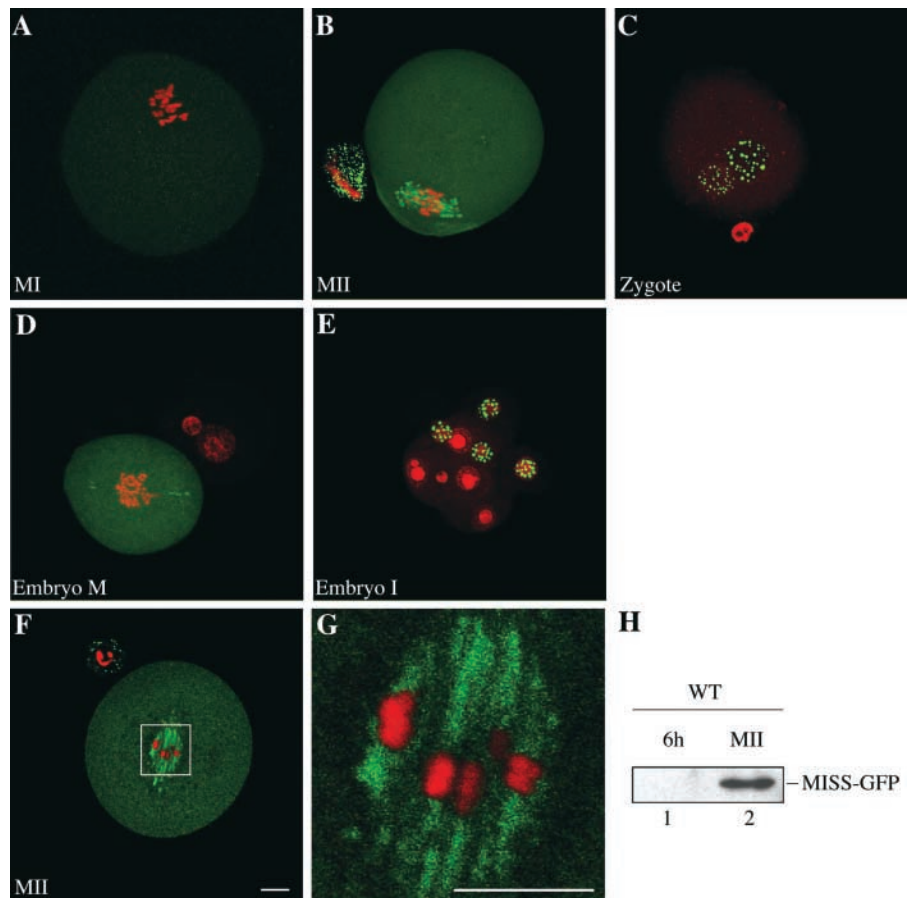
Figure 3. Immunofluorescent staining of MISS in oocytes and early embryos. Groups of 20–30 oocytes at different stages of meiotic maturation were fixed with formaldehyde and further stained with an affinity-purified anti-MISS antibody. All oocytes were analyzed by confocal microscopy using identical settings. (A) Immature oocytes (GV). (B) MI oocyte. (C and D) MII oocyte. (E) Two cell stage embryo. (F) MII oocyte stained with the affinity-purified antibody preincubated with 30 mM of the immunogenic peptide. This experiment was repeated three times. The MISS staining appears in green and chromosomes in red. Bar, 10 μm.

otic maturation. Indeed, the overexpressed MISS-GFP protein is detected by immunoblotting in MII oocytes (Fig. 4 F), and no localization of the MISS-GFP protein is detected in MI oocytes (Fig. 4 A). In MII oocytes, the MISS-GFP localizes in patches along fibers that evoke kinetochore fibers emanating from the chromosomes (Fig. 4, B, F, and G). The MISS-GFP protein also localizes along the spindle in mitotic dividing embryos (Fig. 4 D). After activation and in interphase, the MISS-GFP protein is completely excluded from the cytoplasm and present only in the nucleus, where small dots are observed (Fig. 4, C and E). This result suggests that the NLS present in the protein is functional and involved in a tight regulation of MISS localization.

MISS is unstable in MI and stable in MII

Our anti-MISS antibody was not very sensitive by immunoblotting, as it required 2,000 MII oocytes (one mouse gives

Figure 4. MISS-GFP in mouse oocytes and embryos. Overexpression of MISS-GFP protein after microinjection of RNA coding MISS-GFP into wild-type oocytes, zygotes, and one blastomere of a late two cell stage embryo. Oocytes, zygotes, and embryos were fixed at the indicated stages and analyzed by confocal microscopy. For A–E, the projection of all confocal sections was performed. (A) Oocyte collected in MI. (B) Oocyte collected in MII. (C) Zygote with male and female pronuclei. (D) Two cell embryo injected into one blastomere that is in mitosis (M). (E) Eight cell embryo in interphase (I). (F) Oocyte collected in MII (only one confocal section). (G) Higher magnification of the chromatin area from the oocyte in D. Experiments have been repeated three times. The MISS-GFP staining appears in green and chromosomes in red. Bar, 10 μ m. (H) 20 injected oocytes collected 6 h (lane 1) and 12 h after GVBD (lane 2) were analyzed by immunoblotting using an anti-GFP antibody.



~ 40 oocytes). To analyze more easily the behavior of MISS during mouse meiotic maturation, we microinjected Myc-MISS RNA into mouse immature oocytes and followed the tagged protein during meiotic maturation.

First we checked that the Myc-MISS behaves like the endogenous MISS. Indeed, the exogenous protein could not be detected in immature oocytes, whereas it was present in mature oocytes (Fig. 5 A, lanes 1 and 2). We microinjected chimeric RNA that contained the MISS ORF flanked by the 5' and 3' UTR of the *Xenopus* β -globin gene, thus the translation of the Myc-MISS encoding RNA was not submitted to translational regulations by MISS endogenous 5' and 3' UTR. Using the same construct, we typically observed similar amounts of translation of the exogenous RNA both in immature and mature oocytes when allowed to overexpress for the same amount of time (Fig. 5 A; the levels of expression of Myc-ERK2 are similar in immature and mature oocytes).

Then, we injected a pool of GV oocytes with RNA coding for Myc-MISS and cultured them for the same total amount of time (14 h). We collected them at different stages after meiosis resumption: early MI (2–6 h after GVBD); late MI (8 h after GVBD); or MII (12 h after GVBD). This kind of experiment is possible in mouse oocytes by releasing oocytes after a different length of time spent in medium supplemented with dbcAMP. Meiosis resumption of mouse oocytes occurs spontaneously by removing the oocytes from the ovaries. However, they can be maintained at the GV stage by keeping them in a dbcAMP-containing medium (the dbcAMP mimics a high concentration of AMPc, which

has an inhibitory effect on meiosis resumption). In this way, each sample belongs to a pool of oocytes that were injected at the same time and that had expressed the protein for the same amount of time. We could not detect Myc-MISS protein in early MI oocytes (Fig. 5 B). Myc-MISS accumulates only late during meiotic maturation, around the first polar body extrusion (Fig. 5 B). It suggests that the protein is unstable in MI. We show here that Myc-MISS accumulates rapidly around the first polar body extrusion.

Because the kinetics of Myc-MISS accumulation are similar in wild-type compared with *mos*^{-/-} oocytes (Fig. 5 C), which do not have MAPK activity (Verlhac et al., 1996), the *Mos*.../MAPK pathway does not regulate MISS stability.

To demonstrate that Myc-MISS is indeed unstable in MI due to a high turnover of the protein, we examined its stability in oocytes incubated in nocodazole. In mouse oocytes, cyclin B1 is stabilized by nocodazole treatment, suggesting that its degradation requires intact microtubules (Kubiak et al., 1993). We treated Myc-MISS-injected oocytes with nocodazole for 3 h. As shown in Fig. 5 D, this treatment induces Myc-MISS and cyclin B1 accumulation before polar body extrusion. This shows that MISS is unstable in MI and is degraded by a microtubule-dependent mechanism.

To analyze MISS behavior after parthenogenetic activation, we microinjected Myc-MISS RNA into MII-arrested oocytes. Half of the injected oocytes were activated by ethanol treatment, whereas the other half was not treated. As shown in Fig. 5 E, Myc-MISS accumulates during the MII arrest (lanes 3 and 4), whereas it is unstable after activation (lanes 1 and 2).

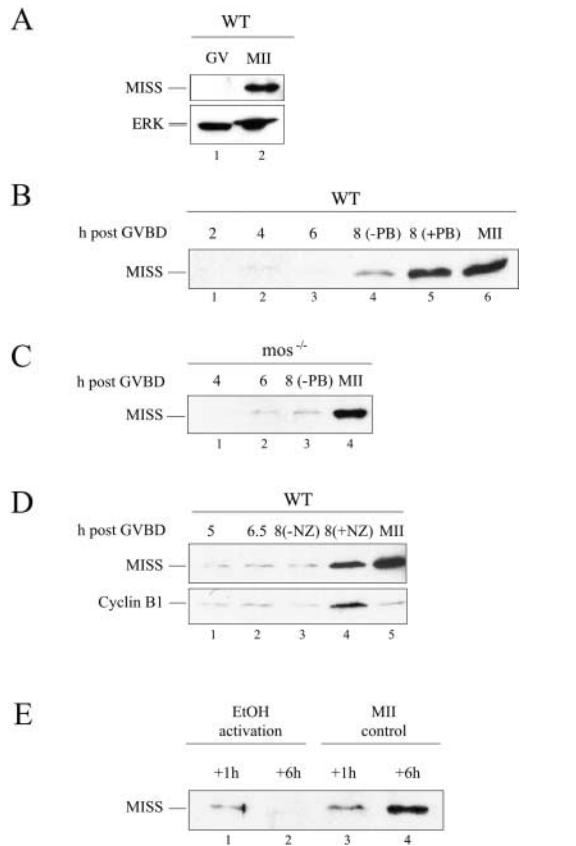


Figure 5. MISS is unstable in MI and activated oocytes and stable in MII oocytes. For A–D, a pool of immature oocytes was injected with RNA and cultured for 14 h. Each sample corresponds to a pool of 20–40 oocytes from the same injection and to oocytes that have expressed the protein for the same amount of time (14 h). Experiments have been repeated three to four times. (A) Overexpression of Myc–MISS protein (top) or Myc–ERK2 (bottom) after microinjection of the corresponding RNA into wild-type immature oocytes. 20 oocytes were collected as immature (lane 1) or mature (12 h after GVBD; lane 2). (B) Myc–MISS-injected wild-type oocytes were collected 2 h (lane 1), 4 h (lane 2), 6 h (lane 3), 8 h (before polar body extrusion; lane 4), 8 h (after polar body extrusion; lane 5), or 12 h (lane 6) after GVBD. (C) Myc–MISS-injected *mos*^{-/-} oocytes were collected 4 h (lane 1), 6 h (lane 2), 8 h (before polar body extrusion; lane 3), or 12 h (lane 4) after GVBD. (D) Myc–MISS-injected wild-type oocytes were collected 5 h (lane 1), 6.5 h (lane 2), 8 h (before polar body extrusion; lane 3), or 12 h (lane 5) after GVBD. Oocytes microinjected with Myc–MISS were also cultured for 5 h after GVBD, incubated for 3 h in nocodazole (NZ; 10 μ M), and collected (lane 4). The top panel is the anti-Myc immunoblot, and the bottom panel is the anti-cyclin B1 immunoblot. (E) Myc–MISS-injected wild-type oocytes were activated by ethanol treatment (lanes 1 and 2) or not (lanes 3 and 4) and further cultured for 1 or 6 h. All samples, except bottom blot in D, were analyzed by immunoblotting using an anti-Myc antibody.

As we observed by immunofluorescence on the endogenous protein, Myc–MISS protein accumulated late in MI. Based on the nocodazole experiment and the behavior of other Myc-tagged proteins in mouse oocytes (i.e., Myc–ERK2; Fig. 5 A; Verlhac et al., 2000b), we conclude that MISS is stabilized only during the MII stage.

Myc–MISS is a substrate of the Mos/.../MAPK pathway
The protein sequence of MISS displays four potential ERK phosphorylation sites and a MAPK docking site. We have

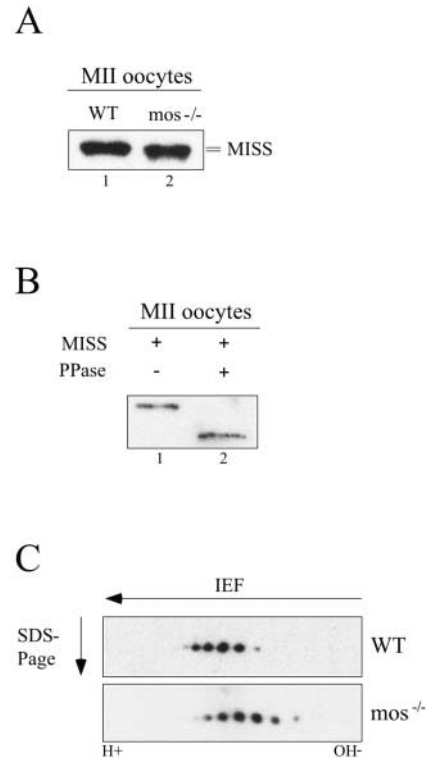


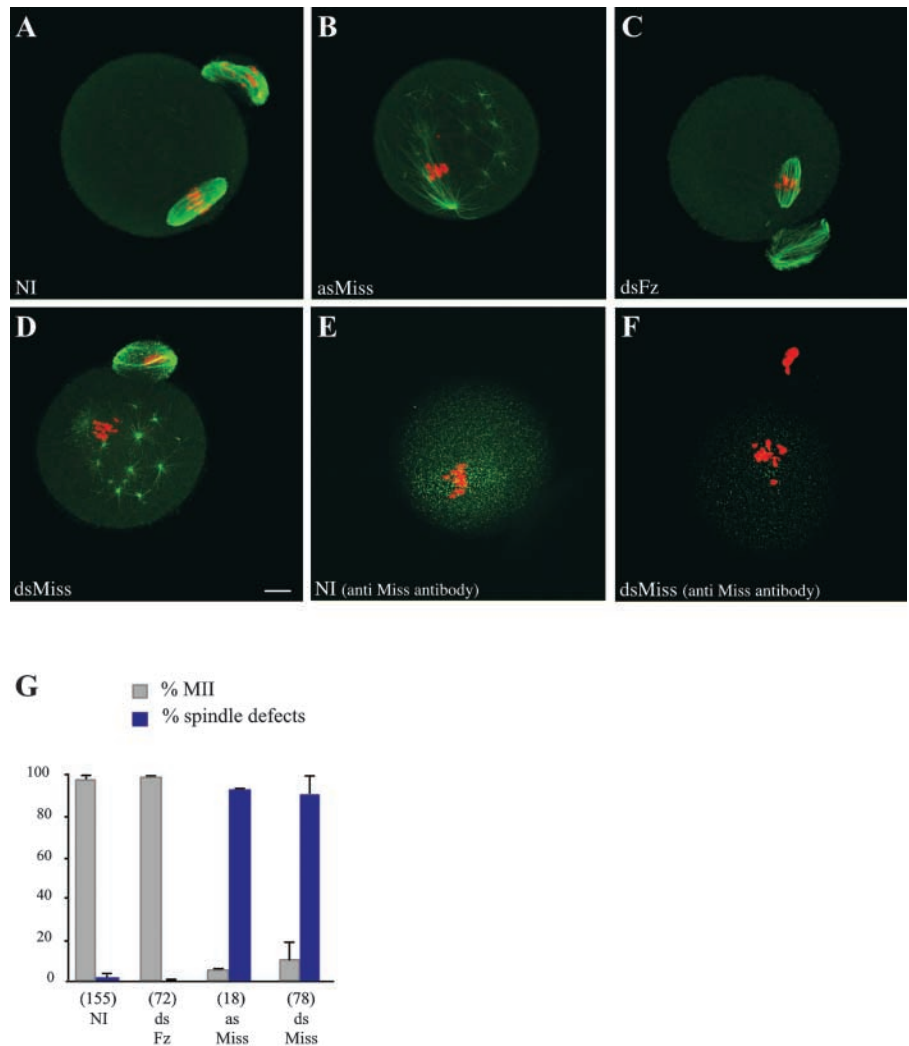
Figure 6. Myc–MISS is a substrate of the Mos/.../MAPK pathway. (A) Myc–MISS-injected oocytes from wild-type (lane 1) or *mos*^{-/-} (lane 2) mice were cultured for 14 h after GVBD and then collected. In the *mos*^{-/-} oocytes (lane 2), the electrophoretic migration of Myc–MISS is faster compared with its mobility in wild-type oocytes (lane 1). (B) In vitro dephosphorylation of Myc–MISS in oocyte extracts. 30 oocytes were injected with RNA encoding Myc–MISS, cultured 14 h after GVBD, collected in water, and incubated without (lane 1) or with 400 U of λ -phosphatase (lane 2). (C) Overexpression of Myc–MISS protein after microinjection of RNA encoding Myc–MISS into immature wild-type or *mos*^{-/-} oocytes. 40 oocytes were collected 14 h after GVBD and were analyzed by 2D gel electrophoresis. The migration of Myc–MISS shifts toward the acidic pole (H⁺) in wild-type oocytes, compared with its migration in *mos*^{-/-} oocytes. We have used an internal control to compare the position of dots (Louvvet-Vallee et al., 2001). Experiments have been repeated three times. All samples were analyzed by immunoblotting using an anti-Myc antibody.

shown that active MAPK can phosphorylate MISS in vitro (unpublished data). To determine whether the protein is an in vivo substrate of MAPK, we checked for its electrophoretic mobility in oocytes from *mos*^{-/-} oocytes. In these oocytes, the migration of Myc–MISS is faster compared with its electrophoretic mobility in wild-type oocytes, reflecting a posttranslational modification of Myc–MISS in wild-type oocytes (Fig. 6 A). Among the posttranslational modifications, we show that at least some of them are associated with phosphorylation. Indeed, treatment of the protein isolated from MII-arrested oocytes with λ -phosphatase induced a downshift of its electrophoretic mobility (Fig. 6 B, compare lanes 1 and 2).

Moreover, the migration of Myc–MISS in 2D gel electrophoresis is shifted to the acidic pole (H⁺) in wild-type oocytes compared with its migration in *mos*^{-/-} oocytes (Fig. 6 C). Because phosphorylations are associated with negative charges, this result suggests that the protein is more phosphorylated in wild-type oocytes compared with *mos*^{-/-} oocytes.

Figure 7. Depletion of MISS mRNA induces severe spindle defects in MII-arrested oocytes.

Immature oocytes were microinjected with asRNA (asMISS) or dsRNA (dsMISS or dsFz) directed against MISS or *Xenopus* frizzled mRNA, collected 14 h after GVBD, fixed, and analyzed by confocal microscopy. For each oocyte, the projection of all confocal sections was performed. (A) Noninjected oocyte (NI) arrested in MII. (B) Oocyte injected with asMISS. (C) Oocyte injected with dsFz (control). (D) Oocyte injected with dsMISS. Microtubules appear in green. (E and F) Oocytes stained with the anti-MISS peptide antibody. (E) Noninjected oocyte in MII. (F) Oocyte injected with dsMISS. For all images, chromosomes appear in red. Bar, 10 μ m. (G) Statistics of the experiment described above. Percent MII, percentage of oocytes presenting a normal bipolar MII spindle; % spindle defects, percentage of oocytes showing monopolar spindle, no spindle, and numerous asters in the cytoplasm. The numbers in brackets correspond to the total number of oocytes analyzed.



Altogether our results show that Myc-MISS is an *in vivo* substrate of the *Mos*/.../MAPK pathway.

Interfering with endogenous MISS mRNA induces severe spindle defects in MII-arrested oocytes

To determine the potential role of MISS during mouse meiotic maturation, we decided to interfere with endogenous mRNA expression. Three different types of molecules were used. First, asRNA (asMISS) or dsRNA (dsMISS) directed against the full-length ORF were microinjected into immature oocytes. The microinjection of either asRNA or dsRNA directed against endogenous MISS mRNA had no effect on GVBD or first polar body extrusion (data not shown). However, both injections induced severe spindle disorganization in MII-arrested oocytes. As shown in Fig. 7, B and D, the MII spindle loses its bipolarity and numerous asters appear in the cytoplasm. This phenotype is visible right after the MII spindle is formed (13 h after GVBD). It is never observed in noninjected oocytes or in oocytes injected with an irrelevant dsRNA at the same concentration and directed against *Xenopus* frizzled 7 (Fig. 7, A and C). As expected, the injection of dsMISS induced a disappearance of MISS endogenous protein in MII oocytes (Fig. 7, compare E and F). Moreover, this phenotype was highly reproducible, as it was observed in >90% of the 96 asMISS- or dsMISS-injected

oocytes (Fig. 7 E). Interestingly, this phenotype is similar to the one observed in *mos*^{-/-} oocytes, which arrest in metaphase III (MIII) with monopolar spindles after second polar body extrusion (Fig. 8 F; Verlhac et al., 1996).

To prove that the spindle phenotype we observed was specific, we then microinjected morpholino oligonucleotides directed against 25 bp upstream of the first ATG (Summerton and Weller, 1997). Again we observed the same phenotype after injection of morpholino directed against MISS endogenous mRNA (Fig. 8 C), but not after injection of the same morpholino presenting five mismatches, as recommended by the manufacturer (Fig. 8 A). The spindle defects observed after injection of the morpholino oligonucleotide were reproducible and were observed in 63% of the cases (Fig. 8 G). We could further show that these defects were specific by rescuing them with an RNA encoding Myc-MISS, which does not contain the 5' UTR sequence and is therefore not targeted by the morpholino. The same oocytes were first injected with control morpholino or anti-MISS morpholino, and then were injected with Myc-MISS-encoding RNA. As shown in Fig. 8, B and D, the injection of Myc-MISS alone or the coinjection of the control morpholino together with Myc-MISS had no effect on spindle organization. However, the injection of Myc-MISS restored normal MII spindle organization (Fig. 8,

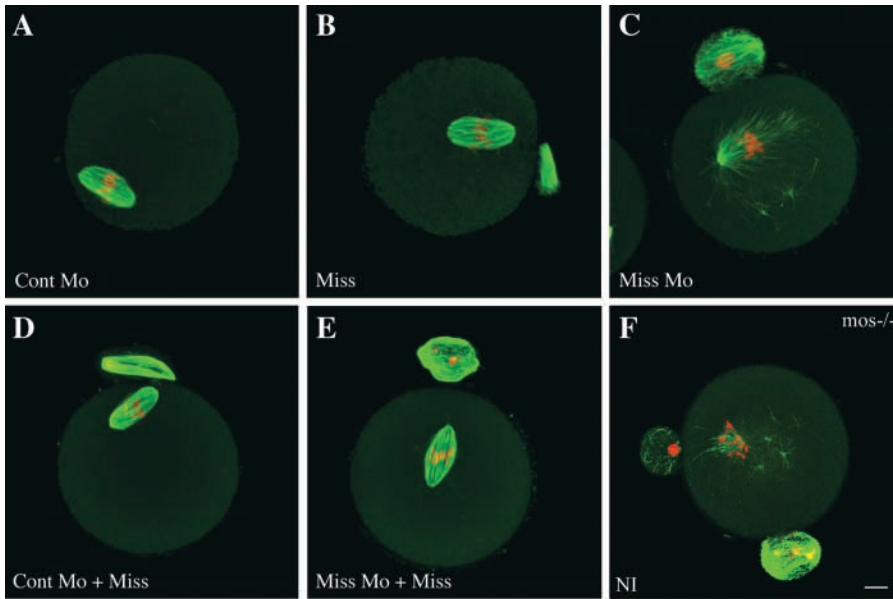
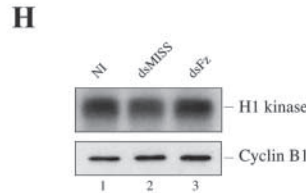
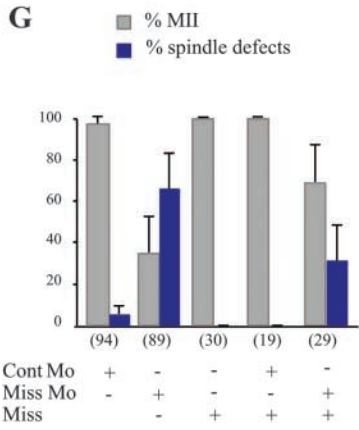


Figure 8. Myc–MISS rescues the depletion of endogenous MISS mRNA after morpholino injection. Immature oocytes were microinjected, collected 14 h after GVBD, fixed, and analyzed by confocal microscopy. For each oocyte, the projection of all confocal sections was performed. (A) Oocyte injected with MISS morpholino containing five mismatches (Cont Mo) as a control. (B) Oocyte injected with Myc–MISS RNA (MISS). (C) Oocyte injected with MISS morpholino (MISS Mo). (D) Oocyte coinjected with both control morpholino and Myc–MISS RNA (Cont Mo+MISS). (E) Oocyte coinjected with both MISS morpholino and Myc–MISS RNA (MISS Mo+MISS). (F) Noninjected (NI) *mos*^{-/-} oocyte arrested in MIII with a monopolar spindle. Microtubules appear in green and chromosomes in red. Bar, 10 μ m. (G) Statistics of the experiment described above. Percent MII, percentage of oocytes presenting a normal bipolar MII spindle; % spindle defects, percentage of oocytes showing monopolar spindle, no spindle, and numerous asters in the cytoplasm. The numbers in brackets correspond to the total number of oocytes analyzed. (H) Histone H1 kinase activity and amount of cyclin B1 in treated oocytes. Oocytes either noninjected (NI; lane 1) or injected with dsMISS (lane 2) or dsFz (lane 3) were collected 12 h after GVBD and analyzed for histone H1 kinase activity (top) and amount of cyclin B1 (bottom).



E and G). The coinjection of anti-MISS morpholino with Myc–MISS RNA induced ~ 2.5 times less oocytes with MII spindle defects than injection of the anti-MISS morpholino alone. MII spindle destabilization in the treated oocytes was not due to cyclin B1 degradation or global drop in histone H1 kinase activity, as shown in Fig. 8 H.

The fact that we observed the same phenotype using three different types of molecules directed against endogenous MISS mRNA, and not with control molecules, is a strong argument in favor of the specificity of the observed spindle defects. This is further demonstrated by the rescue experiment using Myc–MISS RNA in morpholino-injected oocytes.

Altogether our results using three different approaches demonstrate that MISS maintains a bipolar MII spindle during the CSF arrest of mouse oocytes.

MISS does not induce mitotic spindle destabilization in early embryos

Considering the severe phenotypes observed on MII spindles, we can assume that if such spindle defects are observed after injection of dsMISS into early embryos, they should block cell cycle progression. Indeed, similar defects are observed in *mos*^{-/-} oocytes (Fig. 8 F) and induce an MIII arrest. To test this hy-

pothesis and therefore see whether MISS also stabilizes mitotic spindles, we microinjected dsMISS into either zygotes or two cell embryos and checked whether cell cycle progression was inhibited. The injection of dsMISS into zygotes does not block the division from the one to two cell stage: 99% ($n = 38$) of dsMISS-injected zygotes undergo the one to two cell division like the control ($n = 36$) dsFz-injected ones. Also, dsMISS injection does not block the two to four cell division: 79% ($n = 68$) of dsMISS-injected two cell blastomeres divided to four cell blastomeres, comparable to 81% ($n = 70$) of dsFz-injected blastomeres. We checked that our dsMISS were indeed efficient by coinjecting them together with MISS–GFP RNA (Fig. 9). As for zygotes in interphase (Fig. 4 C), the MISS–GFP protein accumulated in the nuclei of the injected blastomeres (Fig. 9 B). However, the MISS–GFP accumulation was very weak when coinjected with dsMISS, compared with its coinjection with dsFz (Fig. 9, B and D). We confirmed this by immunoblotting with an anti-GFP antibody (Fig. 9 E). The MISS–GFP protein accumulated in dsFz- but not in dsMISS-injected blastomeres.

These experiments demonstrate the specificity and high efficiency of our dsMISS; it can almost completely abolish expression of a protein from an abundant exogenous and normally overexpressed RNA. These experiments are consistent

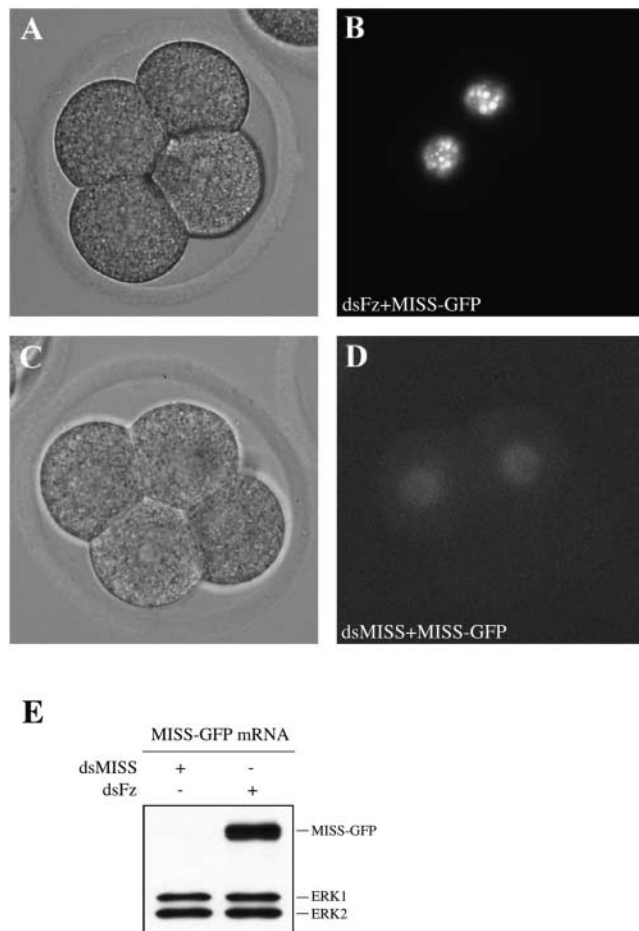


Figure 9. Injection of dsMISS does not block early mitotic divisions. Late two cell embryos were coinjected into one blastomere either with dsRNA against *Xenopus* frizzled 7 (dsFz; A and B) or dsRNA against MISS (dsMISS; C and D) together with the MISS-GFP RNA, and were collected after one division. Live embryos were first observed using a video microscope equipped with a CCD camera (transmitted light on the left; GFP staining on the right), and then embryos were analyzed by immunoblotting using an anti-GFP (top) and an anti-ERK (bottom) antibody (E). The anti-ERK immunoblot serves as a loading control.

with the lack of endogenous MISS staining observed in early mouse embryos and suggest that MISS does not regulate early mitotic divisions.

Discussion

Construction of the cDNA library and isolation of MISS

When we started this project, two two-hybrid screens had been performed using MAPK as a bait (Cook et al., 1996; Waskiewicz et al., 1997). However, no screen had been performed with a mammalian library prepared from synchronized cells where MAPK is known to play an important role. We therefore chose mouse immature oocytes for construction of our cDNA library. These oocytes do not contain mRNA involved in the support of the whole development, as is the case for *Xenopus* oocytes, but they present only mRNA essential for supporting meiotic maturation as well as the first zygotic division. Despite the low amount of RNA present in a mouse oocyte (~1 pg of polyA per oocyte), we

were able to construct a cDNA library. To our knowledge our cDNA library is the sole library of this kind available. It provides an opportunity for the functional screening and discovery of mouse genes involved in meiotic maturation as well as early development.

From the screen performed using ERK2 as bait, we isolated a novel protein, MISS, to which, except for a potential human homologue, we cannot find any obvious homologues in the databases. MISS is very rich in proline residues in its NH₂-terminal end and has four potential ERK2 phosphorylation sites, a perfect PEST sequence, and a bipartite NLS that contains a potential MAPK docking site. We confirmed biochemically the two-hybrid interaction by showing that MISS coimmunoprecipitates with endogenous MAPK.

We show by RT-PCR that the mRNA is present in immature oocytes as well as early mouse embryos. We also show by immunoblotting and immunofluorescence using an affinity-purified antipeptide antibody that MISS protein is present in mouse mature oocytes.

MISS is stabilized only in MII and is phosphorylated by the *Mos*/.../*MAPK* pathway

Both by immunoblotting and immunofluorescence, we show that although the mRNAs are present in immature oocytes, MISS protein is absent from these oocytes. The endogenous protein starts to accumulate late in MI, around the first polar body extrusion. RNA encoding MISS, fused either to a Myc epitope in an NH₂-terminal position or to a GFP epitope in a COOH-terminal position, cannot induce MISS accumulation in GV or early MI oocytes, whereas RNA encoding Myc-ERK2 is typically associated with similar accumulation of Myc-ERK2 in GV and MII oocytes. Moreover, treatment of injected oocytes with nocodazole induces accumulation of the protein in MI, where it normally does not accumulate. Altogether, these data show that MISS is unstable in MI. The stabilization of the protein occurs in MII, after the first polar body extrusion. After parthenogenetic activation, the Myc-MISS protein does not accumulate and is, therefore, probably degraded. This observation is consistent with the fact that we cannot detect endogenous MISS in early embryos by immunofluorescence. The regulation of MISS stability during meiosis is in agreement with the presence of a true PEST sequence in the protein.

The activity that is involved in MISS degradation during MI and disappears in MII does not depend on the *Mos*/.../*MAPK* pathway because Myc-MISS also accumulates in *mos*^{-/-} oocytes.

From studies in *Xenopus* oocytes, we know that MISS is phosphorylated by active MAPK in vitro (unpublished data), and we show that in vivo, MISS is partly phosphorylated by the *Mos*/.../*MAPK* pathway. First, we show that MISS is hyperphosphorylated in MII oocytes. Indeed, both endogenous and Myc-tagged MISS migrate at an apparent molecular weight much higher than the calculated one (45 instead of 29 kD), suggesting the presence of posttranslational modifications. Second, Myc-MISS protein migrates as discrete spots in 2D gel electrophoresis, which suggests different phosphorylation states. Third, treatment of Myc-MISS from MII oocytes with λ-phosphatase induces a severe downshift of the protein. Eventually, we show by 1D and

2D electrophoresis that Myc–MISS is in part phosphorylated by the Mos/.../MAPK pathway.

As MISS is stable only in MII, it is reasonable to assume that the protein is regulated in two steps: (1) stabilization, which is independent of the Mos/.../MAPK pathway but might depend on phosphorylation events by other kinases, and, most likely, (2) full activation through phosphorylation by the Mos/.../MAPK pathway. This implies that the protein is mainly active in MII, when CSF activity is present (Masui and Markert, 1971).

MISS is localized in dots on the MII spindles and disappears after fertilization

The endogenous MISS accumulates in dots on the MII spindles. Also, a MISS–GFP fusion protein shows a strong localization of the protein as bigger patches along fibers that evoke kinetochore microtubules of the MII spindles. In interphase, although we cannot detect the endogenous MISS protein, the MISS–GFP protein concentrates into dots inside the nucleus and is excluded from the cytoplasm. This suggests that the NLS present in the protein is functional.

We cannot explain why the MISS–GFP protein accumulates in early embryos when the endogenous protein does not. One explanation could be that the activity that degrades MISS in GV, early MI, and activated oocytes is no longer present in early embryos, and therefore allows accumulation of an exogenous MISS–GFP protein, whereas it does not allow its accumulation in GV or early MI oocytes. This would imply that a mechanism specific to meiotic maturation controls MISS stability, and this mechanism disappears in early embryos. Alternatively, MISS could undergo other post-translational modifications during early development that prevent its recognition by the antipeptide antibody.

Nonetheless, we believe that MISS is no longer synthesized during early development. First, we showed that Myc–MISS, which behaves like endogenous MISS and can rescue the depletion of the endogenous protein, is no longer accumulated after parthenogenetic activation. Second, we showed that dsMISS has no effect on the first two mitotic divisions, although we demonstrated its activity on abundant exogenous MISS–GFP RNA.

MISS is involved in MII spindle stability during the CSF arrest of mouse oocytes

The localization of MISS and MISS–GFP to MII spindles is consistent with the phenotypes obtained after asRNA, dsRNA, and morpholino injection. Indeed, interfering with the endogenous mRNA by three different means induces severe spindle defects only in MII oocytes, where the protein is present, stable, and probably active. Moreover, we show that the phenotype is associated with a lack of accumulation of endogenous MISS protein in MII. Oocytes that do not possess MISS protein show disorganized spindles often lacking one pole, or have long microtubules emanating from both poles and numerous cytoplasmic asters, which are likely nucleated by cytoplasmic microtubule organizing centers (Maro et al., 1985). The phenotype is specific, because it is rescued by reintroducing Myc–MISS–encoding RNA into anti-MISS morpholino–injected oocytes. This phenotype is also very consistent with the micro-

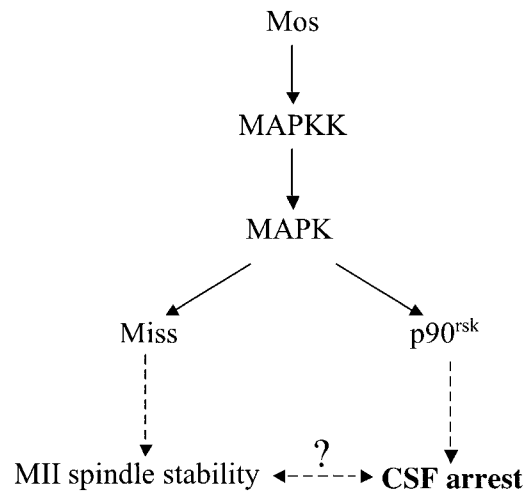


Figure 10. **Proposed model for the role of MISS during mouse meiotic maturation.** See text for details.

tubule defects observed in $mos^{-/-}$ oocytes and with the localization of MAPK at spindle poles of mouse oocytes (Verlhac et al., 1994, 1996). The fact that $mos^{-/-}$ oocytes present some spindle defects could be due to MISS, which is not fully phosphorylated in this strain and probably not active.

Our results suggest that MISS is a spindle-associated protein that is involved in the stabilization of the MII spindle during the CSF arrest. As mentioned in the introduction, it is puzzling that although the Mos/.../MAPK cascade is active in MI, it does not induce CSF arrest in MI. MISS could be an important target of the MAPK cascade that is only stable in MII (Fig. 10). Because we do not observe a significant drop in histone H1 kinase activity as well as a degradation of cyclin B1 in oocytes treated with dsMISS, we think that MISS plays a role in controlling microtubule dynamics, through an interaction with a microtubule associated protein, rather than a direct role in CSF arrest (Fig. 10).

The discovery of this new protein brings some very important insights concerning the CSF arrest in vertebrate oocytes: some substrate(s) of the Mos/.../MAPK pathway is only stable during MII and the Mos/.../MAPK pathway acts not only on the stabilization of maturation-promoting factor (MPF) activity but also on spindle stability. It will be interesting to analyze the relationships between MII spindle stability and CSF arrest.

Materials and methods

Collection and culture of mouse oocytes

Immature oocytes arrested at prophase I of meiosis were obtained by removing ovaries from 11-week-old OF1(WT) and $mos^{-/-}$ female mice. Oocytes were removed and cultured as previously described (Verlhac et al., 1996). Immunofluorescence was performed as previously described (Polanski et al., 1998). Nocodazole was diluted in the culture medium at 10 μ M. Activation by ethanol was performed as previously described (Kubiak, 1989).

Preparation of the mouse oocyte cDNA expression library

Immature oocytes were removed as previously described (Verlhac et al., 1996), collected in sterile PBS, frozen immediately in dry ice, and stored at -80°C . Samples corresponding to 1,200 oocytes were lysed by freezing and thawing. RNA was prepared using the Rneasy Mini Kit (QIAGEN). The genomic DNA was digested by adding 2 U of RQ1 Dnase (Promega) and treating with 40 U of RNase inhibitor (Promega). The RNA was precipi-

tated with ethanol and resuspended in water treated with 0.1% diethylpyrocarbonate (H₂O-DEPC). Double-stranded cDNA was synthesized from RNA using a commercial cDNA synthesis kit (SMART PCR cDNA Synthesis Kit; CLONTECH Laboratories, Inc.), as recommended by the manufacturer, and using 5'-AAGCAGTGGTAACAACGCAGAGTGAATTCGCGGG and 5'-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAG(T)₁₈ primers for the first strand cDNA synthesis. PCR amplification was performed as described in the cDNA synthesis kit using 5'-AAGCAGTGGTAACAACGCAGAGTGAATTCGCGGG and 5'-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTT primers. The cDNAs between 500 pb and 1 kb were gel purified and cloned into pYESTrp (Invitrogen) at EcoRI and XhoI sites. We estimated that the cDNA library consisted of $\sim 3 \times 10^6$ transformants.

Two-hybrid screen

A two-hybrid screen was performed using the Hybrid Hunter Kit (Invitrogen). The bait constructs were generated by PCR amplification from plexA-ERK2WT and plexA-ERK2KD (Waskiewicz et al., 1997) using 5'-TGAC-TAGGATCCGATGGCGGCGCGCGG and 5'-TGCATCGAGTTAAG-ATCTGTATCCTGG primers and cloned as a BamHI/XhoI fragment into pEG202. plexA-Su(Fu) (mouse suppressor of fused) was a gift of A. Plessis (Institut Jacques Monod, Paris, France). We thank J. Cooper for the gift of the pLexA-ERKWT and plexA-ERK2KD plasmids.

The pEG202-lexA-ERK2WT construct was introduced into the yeast EGY48 (*MAT α ura3 trp1 his3 6lexAop-LEU2*) strain transformed with pSH18-34. pEG202-lexA-ERK2WT was tested for spontaneous activation of the LEU2 and lacZ reporter genes. The EGY48 strain transformed with pEG202-lexA-ERK2WT and pSH18-34 was further transformed with the cDNA library cloned into pYESTrp. We screened 4×10^6 transformants.

The two-hybrid tests of specificity were performed using different baits in the strain RFY206 mated with the strain EGY48 containing the potential positive clones.

RT-PCR assay

RT-PCR was processed as previously described (Ledan et al., 2001). PCR amplification was performed using 5'-ATGTATCCCTTCATCCCTCCA and 5'-TCATTTAGACCCTTCACTTTC primers.

Plasmid construction and in vitro synthesis of RNA

pRN3Myc-MISS was constructed by RT-PCR amplification from mouse ovaries. Total RNA was extracted using the Rneasy Mini Kit (QIAGEN). The reverse transcription was performed on 500 ng of RNA. The PCR amplification was done on 50 ng of RNA/DNA using 5'-ATGCGAATTCAGAATGTATC-CCTTCATCCCT and 5'-ATGCGCGGCGCGCTTAAATTAAGTGTATTA primers. The 814-bp PCR product was then cloned into pRN3Myc₂. pRN3MISSGFP was obtained by PCR subcloning at XhoI/EcoRI sites using 5'-ATGCTCGAGATGTATCCCTTCATCCCTCC and 5'-ATGCGAATTC-CTTTAGACCCTTCACTTTC primers. The in vitro synthesis of capped RNA was performed as previously described (Verlhac et al., 2000b).

Microinjection

Microinjection of in vitro-transcribed RNA was performed as previously described (Verlhac et al., 2000b). The sequence of the control morpholino oligonucleotide is 5'-CATACTTCTGCTGCTGCCCGTAG and the sequence of the anti-Miss morpholino oligonucleotide is 5'-CATTCTGTG-TGCTGCTCCGAAG.

Coimmunoprecipitation in *Xenopus* oocyte extracts

Xenopus oocyte microinjection as well as extraction were performed as previously described (Gavin et al., 1999). For the immunoprecipitation of xp42^{mpk1}, we used an anti-ERK2 antibody conjugated to agarose (Santa Cruz Biotechnology, Inc.). *Xenopus* oocyte extracts (300 μ l) were precleared with 20 μ l of protein A coupled to agarose beads for 30 min at 4°C. The cleared extracts were then incubated for 2 h at 4°C with the antibody coupled to agarose beads. The beads were washed four times in lysis buffer supplemented with 150 mM NaCl and then processed for immunoblotting.

Dephosphorylation assay

Just after collection and lysis, oocytes were incubated with or without 400 U of λ -phosphatase (New England BioLabs, Inc.) in λ -phosphatase buffer at 37°C for 1 h and then processed for immunoblotting.

2D gel electrophoresis

Oocytes were stored at -80°C in water containing 1 μ M okadaic acid (LC Laboratories) and 80 μ M β -glycerophosphate. They were then processed as previously described (Louvet-Vallee et al., 2001).

Immunoblotting

Oocytes at the appropriate stage of maturation were collected in sample buffer (Laemmli, 1970) and heated for 3 min at 100°C. We used the following antibodies: an affinity-purified anti-MISS antibody directed against the peptide TMLHVNSQHESEGSK; the 9E10 monoclonal antibody (sc-40; Santa Cruz Biotechnology, Inc.) for Myc-tagged MISS; the monoclonal antibody (no. 1814460, Boehringer) against GFP-tagged MISS; the monoclonal antibody (no. M3530; Dako) against cyclin B1; and the anti-ERK antibody (sc-94; Santa Cruz Biotechnology, Inc.).

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