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The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes

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ABSTRACT **Mos is an upstream activator of mitogen-activated protein kinase (MAPK) and, in mouse oocytes, is responsible for metaphase II arrest. This activity has been likened to its function in *Xenopus* oocytes as a component of cytostatic factor. Thus, Mos-deficient female mice ($MOS^{-/-}$) are less fertile and oocytes derived from these animals fail to arrest at metaphase II and undergo parthenogenetic activation [Colledge, W. H., Carlton, M. B. L., Udy, G. B. & Evans, M. J. (1994) *Nature (London)* 370, 65–68 and Hashimoto, N., Watanabe, N., Furuta, Y., Tamemoto, H., Sagata, N., Yokoyama, M., Okazaki, K., Nagayoshi, M., Takeda, N., Ikawa, Y. & Aizawa, S. (1994) *Nature (London)* 370, 68–71]. Here we show that maturing $MOS^{-/-}$ oocytes fail to activate MAPK throughout meiosis, while $p34^{cdc2}$ kinase activity is normal until late in metaphase II when it decreases prematurely. Phenotypically, the first meiotic division of $MOS^{-/-}$ oocytes frequently resembles mitotic cleavage or produces an abnormally large polar body. In these oocytes, the spindle shape is altered and the spindle fails to translocate to the cortex, leading to the establishment of an altered cleavage plane. Moreover, the first polar body persists instead of degrading and sometimes undergoes an additional cleavage, thereby providing conditions for parthenogenesis. These studies identify meiotic spindle formation and programmed degradation of the first polar body as new and important roles for the Mos/MAPK pathway.**

The *mos* protooncogene encodes a protein serine/threonine kinase (1) and Mos is expressed at high levels in oocytes undergoing meiotic maturation (2, 3). In *Xenopus* oocytes, Mos has been shown to function as a meiotic initiator (4, 5) and an active component of cytostatic factor (CSF) (6), an activity that is responsible for the arrest of an unfertilized egg at metaphase II of meiosis (7). More recently, mitogen-activated protein kinase (MAPK), which is highly activated throughout oocyte maturation (8–12), has been identified as one of the major downstream targets of Mos (13–17). Mos has been implicated in the activation and stabilization of $p34^{cdc2}$ kinase as maturation promoting factor (MPF) (4, 18–20), and evidence for the mutual dependency between MPF and the Mos/MAPK pathway in *Xenopus* oocytes has been reported (8, 21, 22).

More recently, it has been shown that oocytes from mice homozygously deficient in *mos* ($MOS^{-/-}$) fail to arrest at metaphase II and undergo parthenogenetic activation (23, 24). These observations clearly demonstrated that Mos is an active component of CSF, but left unclear whether Mos functions prior to metaphase II arrest as it does in *Xenopus*.

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We generated $MOS^{-/-}$ mice and confirmed previous reports (23, 24) that oocytes from these animals fail to arrest at metaphase II and instead undergo parthenogenetic activation. Also we show that maturing oocytes from $MOS^{-/-}$ mice fail to activate MAPK, while $p34^{cdc2}$ kinase activation is normal until metaphase, when it decreases prematurely. Moreover, in $MOS^{-/-}$ oocytes, we observe that the first polar bodies can be abnormally large and sometimes undergo an additional cleavage instead of undergoing rapid degeneration. Thus, in addition to CSF activity, Mos/MAPK are required for regulating the size and degradation of the first polar body.

MATERIALS AND METHODS

Generation of $MOS^{-/-}$ Knockout Mice. A replacement-type targeting vector was constructed by inserting the neomycin-resistance (*neo*) gene into the *Hpa*I restriction site in the *mos* coding region of a mouse genomic DNA fragment (25). The resulting construct contained about 2 kb and 4 kb of *mos* genomic sequence upstream and downstream of the *neo* gene, respectively. A thymidine kinase cassette was also introduced outside the genomic sequence, as a negative selectable marker. The targeting construct was introduced by electroporation into CJ7 embryonic stem cells and selection was performed as described elsewhere (26). Southern blot analysis of DNAs from the G418/fialuridine-resistant clones was performed to identify homologous recombinants by using a 5′ probe external to the targeting vector. The homologous recombination events were identified by the diagnostic increase in size of the wild-type 7-kb *Hin*dIII fragment to an 8.6-kb *Hin*dIII fragment due to the *neo* sequence insertion. Recombinant embryonic stem clones containing the predicted rearranged band were obtained at a frequency of 1:10. Three embryonic stem cell lines with *mos*-targeted clones that had been injected into C57BL/6 blastocysts generated chimeras transmitting the mutated *mos* allele to the progeny. Breeding of two $MOS^{+/-}$ mice gave rise to homozygous mutant mice at the expected frequency.

Oocyte Collection and Culture. To obtain immature oocytes, 3- to 4-week-old female mice were injected intraperitoneally with 5 units of pregnant-mare-serum gonadotrophin. Cumulus-enclosed immature oocytes were isolated from the ovaries 45–48 hr later and cultured in modified Whitten's medium containing 0.4% bovine serum albumin (BSA) at

Abbreviations: MAPK, mitogen-activated protein kinase; CSF, cytostatic factor; MPF, maturation promoting factor; DAPI, 4′,6-diamidino-phenylindole; MI, period 7 hr after meiotic initiation; MII, period 14 hr after meiotic initiation. IBMX, 3-isobutyl-1-methylxanthine.

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38.5°C in humidified 5% CO_2/95% air (27). The oocyte collection medium was supplemented with 0.1 mM 3-isobutyl-1-methyl-xanthine (IBMX) to inhibit resumption of meiotic maturation (27). The oocytes were then washed in IBMX-free medium and cultured.

Immunofluorescence and *in Vitro* Kinase Assay. After removing the zona pellucida with acidic tyrode solution (pH 2.5), oocytes were fixed with 1.8% paraformaldehyde in PBS for 40 min at room temperature and permeabilized with 1% Triton X-100 in PBS for 20 min. Oocytes were then washed with 0.1% Tween 20 in PBS for 20 min and incubated with PBS containing 3% BSA, 10% goat serum, and 0.1% Tween 20 (blocking solution) for 1 hr at 37°C. The oocytes were incubated with anti-tubulin antibody (YL½, Accurate Chemicals) at a 1:40 dilution and then incubated with fluorescein isothiocyanate-conjugated secondary antibody at 1:20 dilution. For both primary and secondary antibodies, incubation was performed for 1 hr at 37°C. Oocytes were co-stained with 4′,6-diamidinophenylindole (DAPI) for visualization of DNA (27). Immunostained oocytes were examined using a Zeiss 310 confocal laser scanning microscope (Thornwood, NJ).

Histone H1 kinase and MAPK activities were measured as described (27).

RESULTS AND DISCUSSION

Maturing MOS$^{-/-}$ Oocytes Lack MAPK Activity. Since Mos is a potent activator of the MAPK pathway (13–17), we asked whether MAPK was activated in the MOS$^{-/-}$ oocytes. Normally, after removal of IBMX, MAPK activity is detected as early as 5 hr after initiation of meiotic maturation and remains high throughout maturation (27). Extracts prepared from oocytes at the GV (immature oocytes), MI (7 hr after meiotic initiation), and MII (14 hr after meiotic initiation) stages were tested for MAPK activity by using myelin basic protein as a substrate. In MOS$^{+/+}$ oocytes, high MAPK activity was detected in both MI and MII oocytes (Fig. 1*A*). In contrast, MAPK activity was not detected in the stage MI or MII MOS$^{-/-}$ oocytes (Fig. 1*A*). Thus, MAPK activation during mouse oocyte maturation depends entirely on the presence of Mos. In parallel, we examined p34^{cdc2} kinase activity during oocyte maturation by measuring histone H1 kinase activity (Fig. 1*B*). No obvious differences were observed in the cycling or level of p34^{cdc2} activation between MOS$^{-/-}$ and MOS$^{+/+}$ oocytes, except for the dramatic decrease in activity in MOS$^{-/-}$ oocytes after MII (24 hr). Thus, unlike *Xenopus* oocyte maturation (4, 5, 18, 19, 22), in mouse oocytes, MPF activation and regulation appear to be independent of Mos and/or MAPK and MPF does not activate the MAPK pathway.

Abnormal Formation of the First Polar Body in MOS$^{-/-}$ Oocytes. We next examined MOS$^{-/-}$ oocytes during maturation for possible phenotypic alterations. No differences were observed in the timing of germinal vesicle breakdown (GVBD) between MOS$^{-/-}$ and MOS$^{+/+}$ oocytes (data not shown). However, in some of the MOS$^{-/-}$ oocytes, we observed dramatic abnormalities in the formation of first polar body compared with MOS$^{+/+}$ oocytes. Normally at MI, the spindle

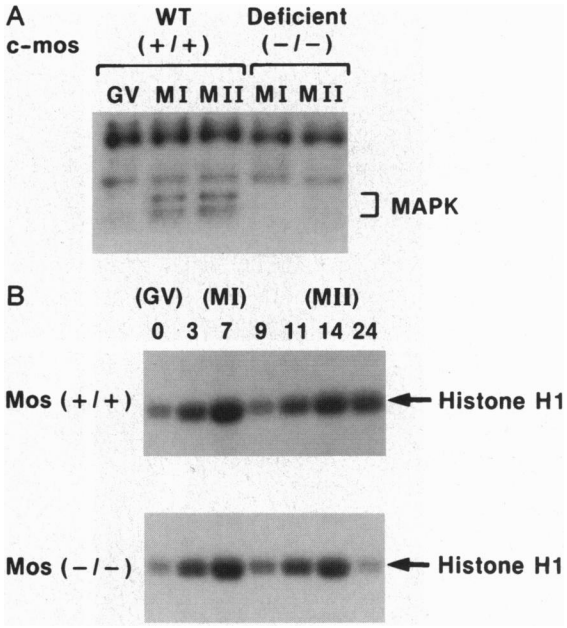

FIG. 1. MAPK and histone H1 kinase activities in MOS$^{-/-}$ oocytes. (*A*) MAPK activity was measured by an in-gel kinase assay using myelin basic protein as substrate (27). Twenty oocytes were selected at 0 hr (GV), 7 hr (MI), and 14 hr (MII) during meiotic maturation. In MOS$^{+/+}$ oocytes, high MAPK activity was detected in both MI and MII oocytes. In contrast, MAPK activity was not detected in the stage MI or MII MOS$^{-/-}$ oocytes. (*B*) Histone H1 kinase activity was measured in total cell extracts (27). Ten oocytes were selected at each indicated time point after meiotic maturation. There were no obvious differences in the cycling kinetics or level of histone H1 kinase activation between MOS$^{+/+}$ and MOS$^{-/-}$ oocytes except for the dramatic decrease in MOS$^{-/-}$ oocytes after MII (24 hr).

is first centrally positioned in the oocyte but then migrates to the cortex. This is a prerequisite for the asymmetric cleavage that produces the small, normally 15–20 μm in diameter, polar body and the remaining larger competent secondary oocyte. At the end of metaphase I, we observed that 20–30% of the MOS$^{-/-}$ oocytes extruded abnormally large polar bodies; half were ≥30 μm in diameter, while the remaining half underwent mitotic-like cleavage producing cells of equal size (Table 1 and Fig. 2). As revealed by anti-tubulin antibody and DAPI staining, we found that these abnormal cell divisions correlated in each case with the failure of the metaphase spindle to properly translocate to the cell surface (Fig. 2). Thus, in the absence of Mos/MAPK, 20–30% of oocytes lose their ability to properly position the spindle apparatus. Despite the abnormal cytokinesis, most, if not all, of these oocytes appear to proceed to MII. However, we do not know whether they are competent for fertilization or embryogenesis although MOS$^{-/-}$ females display reduced fertility (23, 24). By 24 hr the nucleus reforms in ≈90% of these oocytes (data not shown). These results suggest that Mos/MAPK influences the positioning of the metaphase spindle during meiosis and are

Table 1. Abnormal first polar body formation in MOS$^{-/-}$ oocytes

	Total number of oocytes examined	Number of oocytes that extruded first polar body	Polar body phenotypes		
			Normal polar body	Large polar body	Symmetrical division
MOS$^{+/+}$, MOS$^{+/-}$	163	146/163 (90%)	146/146 (100%)	0	0
MOS$^{-/-}$	187	161/187 (86%)	126/161 (78%)*	21/161 (13%)	14/161 (9%)

Oocytes were examined 10 hr after initiation of meiotic maturation for extrusion of the first polar body. The representative polar body phenotypes are shown in Fig. 2; normal (Fig. 2*d*), large (Fig. 2*e*), symmetrical division (Fig. 2*f*). The large polar body has a diameter ≥30 μm. No differences in the frequency of the first polar body extrusion were observed between MOS$^{+/+}$ and MOS$^{+/-}$ oocytes.

*Of the first polar bodies categorized as "normal" in MOS$^{-/-}$ oocytes, ≈30% were slightly larger than MOS$^{+/+}$ and MOS$^{+/-}$ polar bodies (data not shown).

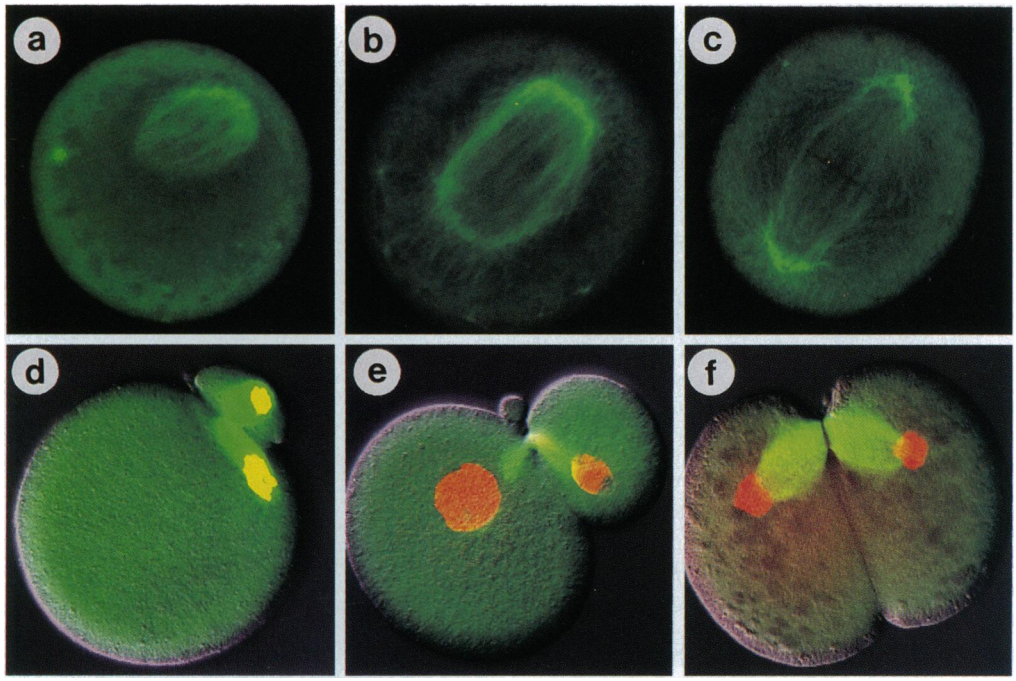

FIG. 2. Abnormal positioning of spindle apparatus and first polar body formation in MOS$^{-/-}$ oocytes. Oocytes were selected before (at 8 hr) (*a–c*) and after (9 hr) (*d–f*) extrusion of the first polar body and stained with anti-tubulin YL½ antibody (green) and DAPI (red). In control MOS$^{+/+}$ oocytes, one spindle pole is juxtapositioned to the membrane (*a*) and a normal-size polar body is extruded (*d*). In contrast, in MOS$^{-/-}$ oocytes, the spindle apparatus is observed in the center rather than at the membrane (*b*, *c*, *e*, and *f*); a large polar body (*b* and *e*) and symmetric cell division (*c* and *f*) are also shown.

consistent with the previously identified role of Mos and MAPK in microtubule reorganization (27–30, 41).

Normally, the microtubule arrays present during prophase disappear as the spindle apparatus begins to form at ≈5 hr after meiotic initiation (data not shown). Immunostaining with anti-tubulin antibody showed that, in MOS$^{-/-}$ oocytes, the microtubule arrays persist through metaphase I and 7 hr after meiotic initiation (Fig. 2 *b* and *c*). MAPK has been implicated in the severing activity of microtubules (29) and in the absence of Mos/MAPK the extensive astral-like microtubule arrays appear to persist and thereby may prevent proper association of the spindle with the inner surface of the oocyte cortex. These data identify spindle positioning as an important new Mos/MAPK function and are consistent with our observations that expression of Mos/MAPK in somatic cells leads to anastral meiotic-like spindles positioned adjacent to the cell membrane that give rise to binucleated cells (28).

First Polar Body Persistence in MOS$^{-/-}$ Oocytes. Another difference we observed in maturing MOS$^{-/-}$ oocytes was the long-term persistence of the first polar body in 90% of the oocytes (Table 2 and Fig. 3). After extrusion, the first polar body is degraded in MOS$^{+/+}$ oocytes by 7 hr, while in MOS$^{-/-}$ oocytes they persist for more than 11 hr (Table 2). Not only do the polar bodies persist, but they also elongate and frequently undergo an additional division (Fig. 3). The altered life span of the first polar body in MOS$^{-/-}$ oocytes suggests that rapid degeneration may be a programmed event that is triggered by Mos/MAPK. We have previously shown that Mos/MAPK trigger apoptosis in somatic cells (13). The involvement of Mos/MAPK in degeneration of the first polar body might serve to explain the significantly longer life span of the second polar body, which is extruded after fertilization when Mos/MAPK are being degraded and inactivated (30). Thus, our results suggest that another unique function of Mos/MAPK is to cause programmed degeneration of the first polar body which might explain at least one form of parthenogenesis. The presence of parthenogenetically activated MOS$^{-/-}$ oocytes in the follicle before ovulation (24) raises the possibility that they result from an abnormal meiotic division. A similar situation has been observed in oocytes of LT/Sv mutant female mice that arrest and ovulate at metaphase I or II (31, 32) and spontaneously undergo parthenogenetic activation (33, 34) in the ovary (35). Fertilization of primary oocytes from LT/Sv mutant female mice is possible (36). We do not know whether the persisting large polar bodies of MOS$^{-/-}$ oocytes are competent for fertilization, but fertilization of a diploid ovum resulting from an error in the first meiotic division has been proposed as one mechanism responsible for combined placental mosaicism (CPM) (37). CPM occurs in a high percentage of human conceptuses (1–3%) (38) and may be attributed to the acquisition of an extra haploid set of maternal (digynic) chromosomes (39). The loss of Mos/MAPK function and the long-term stability of polar bodies in MOS$^{-/-}$ mice could provide an alternative mechanism for CPM.

The accuracy of chromosome partitioning during oocyte meiotic maturation is a prerequisite for the continuous genetic integrity of the species. We would expect that the alteration observed in MOS$^{-/-}$ oocytes would lead to increased genetic instability; just as we have proposed that inappropriate ex-

Table 2. Comparison of kinetics of the first polar body degeneration in MOS$^{+/+}$, MOS$^{+/-}$, and MOS$^{-/-}$ oocytes

	No. oocytes that retained first polar body/no. total oocytes examined (%)			
	0 hr	3 hr	7 hr	11 hr
MOS$^{+/+}$, MOS$^{+/-}$	75/75 (100%)	70/75 (93%)	9/75 (12%)	0/75 (0%)
MOS$^{-/-}$	68/68 (100%)	68/68 (100%)	61/68* (90%)	61/68[c] (90%)

Oocytes extruded the first polar body between 8 and 9 hr after meiotic maturation. Oocytes were examined 0, 3, 7, and 11 hr after first polar body extrusion. We did not observe differences in the kinetics of first polar body degeneration between MOS$^{+/+}$ and MOS$^{+/-}$ oocytes.
*Among the oocytes retaining first polar bodies, 18/61 (30%) at 7 hr and 29/61 (48%) at 11 hr contained two or more polar bodies.

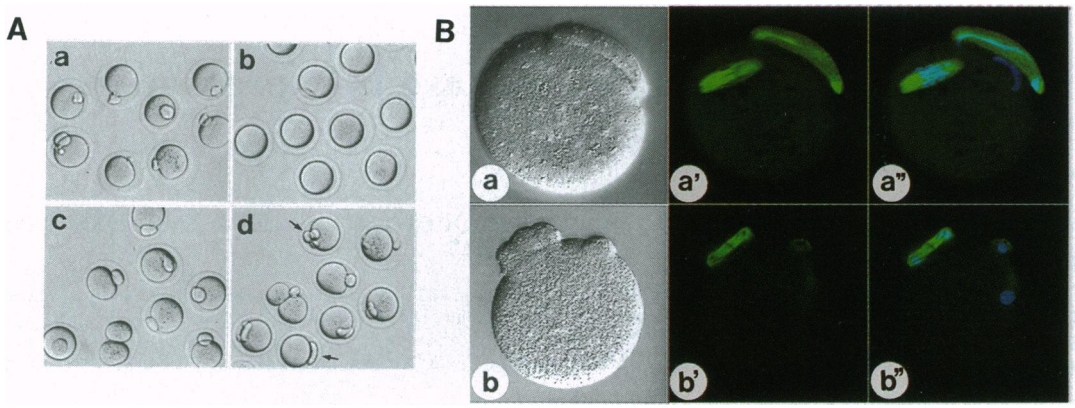

FIG. 3. Persistence of the first polar body in MOS$^{-/-}$ oocytes. (*A*) Both MOS$^{+/+}$ (part a) and MOS$^{-/-}$ (part c) oocytes extrude first polar bodies at 8–9 hr after meiotic maturation. At 7 hr after first polar body extrusion, most of the polar bodies of MOS$^{+/+}$ oocytes are degraded (part b), while those of MOS$^{-/-}$ oocytes (part d) remain intact and also elongate and frequently undergo an additional division (indicated by arrows). (*B*) Two MOS$^{-/-}$ oocytes at 7 hr after first polar body extrusion were stained with anti-tubulin (green) and DAPI (blue). Parts: a and b, Nomarski images; a′ and b′, tubulin; a″ and b″, tubulin/DAPI.

pression of Mos/MAPK in somatic cells increases genetic instability due to aberrant expression of meiotic activities (28).

Note Added in Proof. While this paper was in press we discovered that the article by Verlhac et al. (40) was published in *Development*. This article agrees with our findings.

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