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Report

Involvement of Polo-like kinase 1 in MEK1/2-regulated spindle formation during mouse oocyte meiosis

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Key words: MEK1/2, Plk1, spindle formation, meiosis, mouse oocyte

Our recent studies have shown that MEK1/2 is a critical regulator of microtubule organization and spindle formation during oocyte meiosis. In the present study, we found that Plk1 colocalized with p-MEK1/2 at various meiotic stages after GVBD when microtubule began to organize. Also, Plk1 was able to coimmunoprecipitate with p-MEK1/2 in metaphase I stage mouse oocyte extracts, further confirming their physical interaction. Taxol-treated oocytes exhibited a number of cytoplasmic asters, in which both Plk1 and p-MEK1/2 were present, indicating that they might be complexed to participate in the acentrosomal spindle formation at the MTOCs during oocyte meiosis. Depolymerization of microtubules by nocodazole resulted in the complete disassembly of spindles, but Plk1 remained associated with p-MEK1/2, accumulating in the vicinity of chromosomes. More importantly, when p-MEK1/2 activity was blocked by U0126, Plk1 lost its normal localization at the spindle poles, which might be one of the most vital factors causing the abnormal spindles in MEK1/2-inhibited oocytes. Taken together, these data indicate that Plk1 and MEK1/2 regulate the spindle formation in the same pathway and that Plk1 is involved in MEK1/2-regulated spindle assembly during mouse oocyte meiotic maturation.

Introduction

The accurate segregation of chromosomes during both mitosis and meiosis is essential to ensure the propagation of cells and species. Segregation errors during meiosis lead directly to birth defects, and segregation errors during mitosis in somatic cells contribute to the development and progression of cancer.[1] This critical event in the cell cycle is driven by a complex superstructure called the spindle, mainly composed of microtubules and centrosomes.[2] During mitosis, centrosomes are the main sites of microtubule polymerization and thus spindle assembly. They translocate to opposite sides of nucleus, where they nucleate microtubules, which are captured and stabilized by the kinetochore of the sister centrosomes of chromosomes after nuclear envelope breakdown, facilitating rapid organization of a bipolar spindle.[3] But in many species, including *Homo sapiens*, *Mus musculus*, *Caenorhabditis elegans* and *Drosophila melanogaster*, only the sperm contributes the centrioles to the zygote; the oocyte itself, once it enters meiosis, lacks centrosomes.[4] Thus, in the oocytes of these organisms, the acentrosomal spindle assembly relies on the self-organization of numerous acentriolar MTOCs (Microtubule organizing centers), which functionally replace centrosomes.[5]

In our previous study, we have revealed that MEK1/2 plays critical roles in regulating the microtubule organization and spindle formation during mouse oocyte meiotic maturation,[6] and this is further confirmed by the work in rat oocytes.[7] While several lines of evidence also showed that MEK1/2 may not directly participate in this event, but regulate some unknown downstream protein kinases.[7,8] Thus we aim to identify this possible new substrate of MEK1/2.

Polo-like kinase, a family of serine/threonine protein kinases, is conserved among organisms from yeast to mammals. Named after the Drosophila *polo* gene originally identified through a recessive maternal effect lethal mutation, Plk homologues have been successively identified in yeast, Xenopus, *C. elegans* and mammals. Mammals express at least three different Plks: Plk1, Plk2 and Plk3. Of these, Plk1 clearly functions during M phase and is most likely to represent the functional homologue of Drosophila *polo*.[9-11] Plk1 has been found to participate in multiple important cellular events, including entry and exit of mitosis,[10,12] DNA checkpoint activation,[13] centrosome regulation,[14] chromosome alignment,[15] chromosome segregation,[16] anaphase-promoting complex regulation,[17] proteasome phosphorylation,[18] cytokinesis[19] and especially the bipolar spindle formation.[20-22] In meiosis, our earlier findings have indicated that Plk1 plays pivotal roles in regulating the microtubule assembly and spindle formation in mouse, rat and porcine oocytes.[23-25] Since both MEK1/2 and Plk1 are shown to localize to the spindle poles and play an essential role in spindle assembly during mouse oocyte meiotic maturation, we hypothesized that Plk1 might be a molecule interacting with MEK1/2 in the regulation of spindle formation.

Here, we investigated the relationship between MEK1/2 and Plk1 by immunofluorescence, coimmunoprecipitation and MEK1/2 inhibitor treatment. The combined data present several lines of evidence showing that Plk1 might be involved in MEK1/2-regulated spindle assembly during mouse oocyte meiosis.

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Results

Plk1 colocalizes with p-MEK1/2 during mouse oocyte meiotic maturation. We have recently shown that MEK1/2, an intermediate protein kinase in the MAPK cascade, serves as an important regulator in microtubule organization and spindle formation during mouse and rat oocyte meiosis, but these functions may be performed neither directly nor through its classic substrate MAPK.[6-8] Since it has been reported that MEK kinase could phosphorylate MEK1 and MEK2 but not cause activation of MAPK, suggesting that MEK1/2 may exert its function in a signaling pathway that is independent of MAPK activation,[26] we intend to search the probable downstream molecules of MEK1/2 in the regulation of spindle formation. Plk1, interacting with a number of proteins required for spindle formation, is chosen as the study candidate.

We first investigated the spatial relationship between Plk1 and p-MEK1/2. Double immunostaining of these two molecules were carried out using the mouse monoclonal anti-Plk1 antibody and rabbit polyclonal anti-phospho-MEK1/2 antibody. As shown in Figure 1, shortly after GVBD, Plk1 foci aggregated in the vicinity of the condensed chromosomes which were scattered in the center of the oocytes. At this stage, p-MEK1/2 followed the same localization as Plk1 and their signals overlapped. By prometaphase I stage, Plk1, colocalizing with p-MEK1/2, began to migrate toward the spindle poles before the chromosomes aligned at the equator of the spindle. As oocytes progressed to metaphase I stage when chromosomes aligned at the equatorial plate, both Plk1 and p-MEK1/2 were coincidently localized to the spindle poles. At metaphase II stage, with the first polar body extrusion, Plk1 and p-MEK1/2 again colocalized at the spindle poles. In our earlier work,[6,24] the single staining of Plk1 and p-MEK1/2 has been shown in mouse oocytes, respectively. The previous localization patterns of these two melocules are consistent with the ones in double staining here, together with the below result of coimmunoprecipitation, we could rule out the possibility that colocalization of Plk1 and p-MEK1 is an artifact.

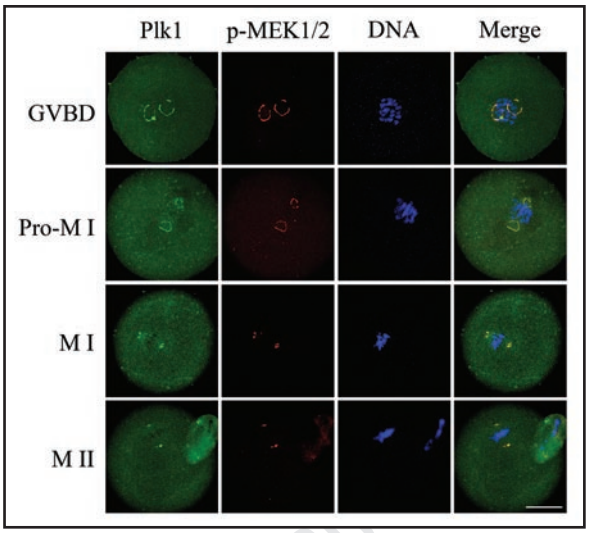

Figure 1. Colocalization of Plk1 and p-MEK1/2 during mouse oocyte meiotic maturation. Oocytes at various stages after GVBD were double stained with antibodies against Plk1 and p-MEK1/2. Green, Plk1; red, p-MEK1/2; blue, chromatin; yellow, overlapping of green and red. GVBD, oocytes at germinal vesicle breakdown; Pro-MI, oocytes at first prometaphase; MI, oocytes at first metaphase; MII, oocytes at second metaphase. Each sample was counterstained with Hoechst 33258 to visualize DNA. Scale bar, 20 μm.

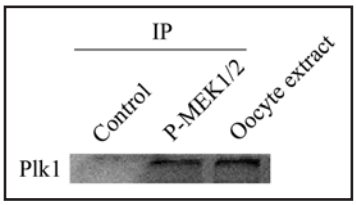

Figure 2. Coimmunoprecipitation of Plk1 and p-MEK1/2 in metaphase I mouse oocyte extracts. Immunoprecipitation experiments shown in the first two lanes were performed using a control irrelevant antibody (anti-α-tubulin) and a rabbit antibody against p-MEK1/2, respectively. In the control, there was no band. The last lane shows immunoblots of mouse oocyte extracts, detected by anti-Plk1 antibody.

Plk1 physically interacts with p-MEK1/2 at metaphase I stage. To further confirm the physical interaction between Plk1 and p-MEK1/2, we immunoprecipitated p-MEK1/2 in metaphase I stage mouse oocyte extracts and then performed the immunoblotting with the anti-Plk1 antibody. As shown in Figure 2, in oocyte extract lane, Plk1 was detected as a specific band in the membrane. In the control lane, immunoprecipitation was carried out with an irrelevant antibody (anti-β-actin) instead of anti-p-MEK1/2 antibody, so no band was detected in the immunoprecipitates when running the immunoblotting using anti-Plk1 antibody. In p-MEK1/2 lane, by contrast, Plk1 could be detected in the immunoprecipitates and appeared at the same positions corresponding to the mouse extract lane. Collectively, the data presented above imply that Plk1 is complexed with p-MEK1/2 to exert the functions during the spindle formation.

Plk1 colocalizes with p-MEK1/2 at the cytoplasmic MTOCs in mouse oocytes. During mitosis, spindle assembly is directed to a large extent by the centrosomes, the main sites of microtubule polymerization. Mouse oocytes, however, lack centrosomes and the microtubules are polymerized at discrete sites in the cytoplasm called MTOCs.[27] A recent report proposes a new model of acentrosomal spindle assembly that relies on the self-organization of numerous acentriolar MTOCs, which functionally replace centrosomes.[5] Therefore, it is interesting to observe whether Plk1 and p-MEK1/2 would be associated at the MTOCs.

To test this, oocytes were treated with taxol, a microtubule-stabilizing agent, to produce the cytoplasmic asters and then triply stained for Plk1, p-MEK1/2 and α-tubulin. In taxol-treated oocytes, after GVBD when microtubule organization initiated, the microtubule fibers were excessively polymerized, leading to the significantly enlarged spindles together with many asters that were discretely distributed throughout the cytoplasm. In this context, p-MEK1/2 signals were present as small dots in the center of these asters and spindle poles, in accord with the staining pattern of Plk1 (Fig. 3).

Plk1 colocalizes with p-MEK1/2 in nocodazole-treated oocytes. Next, to examine the correlation between Plk1 and p-MEK1/2 when normal microtubule organization was disrupted, nocodazole, a microtubule-depolymerizing drug, was used to treat the oocytes. After treatment, the microtubules were completely disassembled and no intact spindles could be observed. In this case, translocation of p-MEK1/2 to the spindle poles was not visible due to its dependence on

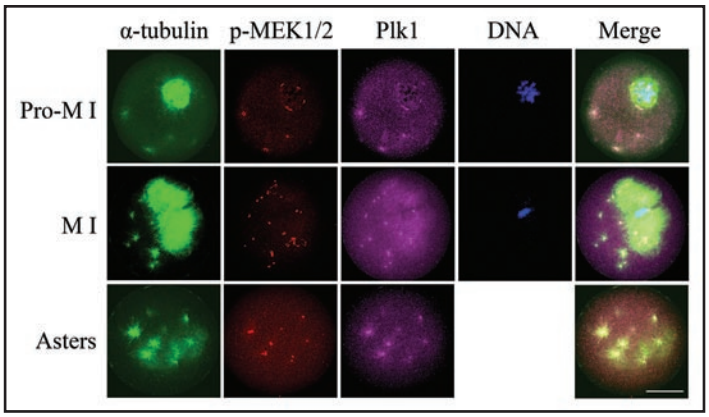

Figure 3. Colocalization of Plk1 and p-MEK1/2 in the MTOCs induced by taxol. Taxol-treated oocytes were triply stained with antibodies against α-tubulin, Plk1 and p-MEK1/2. Green, α-tubulin; red, p-MEK1/2; pink, Plk1; blue, chromatin. Pro-MI, oocytes at first prometaphase; MI, oocytes at first metaphase. Each sample was counterstained with Hoechst 33258 to visualize DNA. Scale bar, 20 μm.

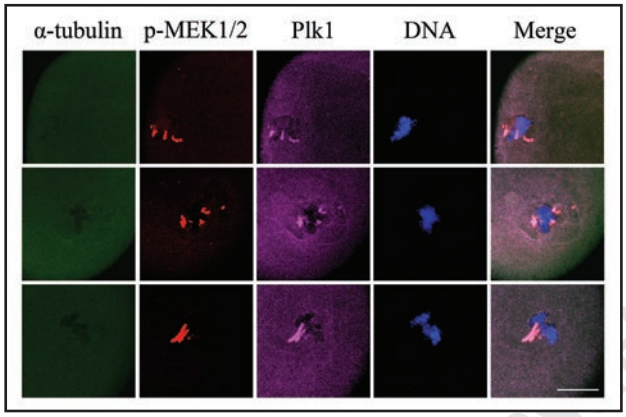

Figure 4. Colocalization of Plk1 and p-MEK1/2 in the oocytes treated with nocodazole. Nocodazole-treated oocytes were triply stained with antibodies against α-tubulin, Plk1 and p-MEK1/2. After treatment, microtubules were completely depolymerized and Plk1 colocalized with p-MEK1/2 in the vicinity of chromosomes. Green, α-tubulin; red, p-MEK1/2; pink, Plk1; blue, chromatin. Each sample was counterstained with Hoechst 33258 to visualize DNA. Scale bar, 10 μm.

the intact spindle microtubules.[28] Instead, p-MEK1/2 amorphously accumulated in the vicinity of chromosomes, colocalizing with Plk1 (Fig. 4), indicating that the association of Plk1 with p-MEK1/2 would not be disrupted even when the spindle is disassembled and that Plk1 might possess the similar mechanism regarding the intracellular translocation as MEK1/2, which needs future confirmation.

U0126 treatment leads to the delocalization of Plk1 and abnormal spindles. Most importantly, we tried to study the hierarchy of Plk1 and MEK1/2 in the regulation of spindle assembly and determine whether Plk1 lies in the downstream of MEK1/2. To this purpose, we treated the oocytes with the U0126, a MEK specific inhibitor, and then asked whether the spindle formation and Plk1 localization would be affected. As shown in Figure 5, in the control group, bipolar spindles were normally formed in the oocytes, and Plk1 were localized to the spindle poles. In U0126-treated group, by contrast, the oocytes exhibited various morphologically defective spindles, including spindles without well-organized poles, spindles with numerous microtubules oppositely emanating from the poles and unformed spindles with astral microtubules as well as many cytoplasmic asters. At the same time, the altered localization of Plk1 was also observed: it translocated from spindle poles to the entire spindle fibers, colocalizing with the α-tubulin and/or β-tubulin. Thus, this observation gives us a hint that mislocalization of Plk1 may be related to the aberrant spindles in MEK1/2-inhibited oocytes.

Discussion

In this report, we demonstrate that Plk1 may be involved in MEK1/2-regulated spindle assembly during mouse oocyte meiosis. It is well known that mitogen-activated protein kinase (MAPK) cascade, one of the most important intracellular signaling pathways, plays pivotal roles in regulating the meiotic cell cycle progression of oocytes.[29-32] However, as an intermediate kinase that acts upstream of MAPK and downstream of MEK kinase, MEK functions are poorly known. Recently, we have reported that MEK1/2 regulates microtubule organization, spindle pole tethering and asymmetric division during mouse oocyte meiotic maturation[6] and that MEK1/2 is a critical regulator of microtubule assembly and spindle organization during rat oocyte meiotic maturation.[7] In the latter study, we also found that when MEK1/2 is inhibited by U0126, there are still some small spindle-like structures present in the cytoplasm after taxol treatment, suggesting that MEK1/2 may be not directly involved in the microtubule organization and spindle assembly, but regulate some unknown downstream protein kinases. This notion is consistently supported by another work that when mouse oocytes are treated with a low dose of U0126, the phosphorylation of MAPK is not changed but the spindle fails to maintain its structures.[8] Therefore, these intriguing findings prompted us to search the possible substrate of MEK1/2 in regulating the spindle formation during oocyte meiosis.

To date, accumulating reports have been shown that Plk1 interacts with numerous molecules and thus participates in a number of cellular processes. The human Plk regulates cdc2/cyclinB through phosphorylation and activation of the cdc25C phosphatase, allowing entry into mitosis.[12] Nlp, identified as a centrosomal Plk1 substrate, plays important roles in microtubule nucleation under the regulation of Plk1 phosphorylation.[33] In prometaphase cells, BubR1 colocalizes with Plk1 at kinetochores of unaligned chromosomes and physically interacts with Plk1, suggesting that Plk1 facilitates chromosome alignment during prometaphase through BubR1.[15] At the transition from metaphase to anaphase, Plk has been shown to be associated with the anaphase-promoting complex (APC) and to regulate ubiquitination, the process that targets proteins for degradation by proteasomes.[18] In addition, Plk can interact with proteasomes and regulate their activity.[18] The characterization of interaction between Plk1 and NudC showed that NudC is a substrate for Plk1 and Plk1 phosphorylation of NudC may influence cytokinesis.[34] Among these multiple functions of Plk1 during the cell cycle progression, we pay more attention to its roles in bipolar spindle formation. In both interphase and mitotic cells, Plk specifically associates with alpha-, beta- and gamma-tubulins, providing a probable molecular basis for the physiological functions of Plk in regulating the cell cycle, particularly in establishing the normal bipolar spindle.[35] Moreover, another finding by the same group also indicates that GTP-binding

protein Ran is a physiological substrate of Plk1 and that Plk1 regulates the spindle organization partially through its phosphorylation on Ran.[36] Accordingly, the fact that Plk1 is required for the bipolar spindle formation during both mitosis and meiosis[21,22,24] and that it can interact with numerous molecules playing essential roles in the spindle formation raises the possibility that Plk1 might interact with MEK1/2 functioning in the spindle assembly during oocyte meiosis.

First of all, the spatial relationship between Plk1 and p-MEK1/2 was examined by immunofluorescent analysis. In our previous work, we found that after GVBD when microtubules begin to polymerize, Plk1 and p-MEK1/2 are detected in the periphery of condensed chromosomes, respectively. By metaphase I and metaphase II stages, when chromosomes align at the equatorial plate and the typical bipolar spindle forms in the cytoplasm, both Plk1 and p-MEK1/2 are observed at the spindle poles.[6,24] Thus, the similar localization pattern of Plk1 and p-MEK1/2 could predict their possible interaction. In the present study, we carried out the double staining of Plk1 and p-MEK1/2 at various developmental stages during mouse oocyte meiosis to directly observe their association. The colocalization of these two molecules during the entire formation of spindles suggests that they might be in the same spindle formation pathway. Next, we run the coimmunoprecipitation to further test their physical interaction. As expected, Plk1 was physically associated with p-MEK1/2, an indication that they form a complex, maybe containing other proteins, to participate in the spindle organization. The similar interaction is also observed between Plk3 and MEK1. Plk3 has been reported to interact with MEK1 during cell cycle and mediate MEK1 function in the Golgi fragmentation.[37]

Subsequently, spindle-perturbing drugs were used to investigate the relationship between Plk1 and p-MEK1/2 when microtubule dynamics was changed, and then triple staining of α-tubulin, Plk1 and MEK1 was performed to observe their localization. After treatment with taxol, oocyte exhibited an enlarged spindle together with a number of cytoplasmic asters. Consistent with the earlier observation,[6] p-MEK1/2 was present in the center of the asters scattered in the cytoplasm. Meanwhile, signals of Plk1 were also detected at these MTOCs, overlapped by p-MEK1/2. In mitosis, the spindle formation relies on the centrosome, but acentrosomal spindle assembly in mouse oocytes during meiosis is mainly dependent on the MTOCs. Therefore, the association of Plk1 with p-MEK1/2 in the center of MTOCs further reveals that the complex containing Plk1 and MEK1/2 plays critical roles in microtubule organization and spindle formation. Additionally, when spindle formation was disrupted by treatment with the microtubule-depolymerizing agent nocodazole, Plk1 and p-MEK1/2 did not migrate to the spindle poles but colocalized in the periphery of chromosomes. Combining these results, we can see that Plk1 is always associated with MEK1/2 no matter how the spindle changes.

The above observations just provide evidence showing that Plk1 and MEK1/2 work in the same pathway to regulate the meiotic spindle formation. To address the question whether MEK1/2 lies in the upstream of Plk1 in the regulatory cascade, we employed U0126 to treat the oocytes and then checked the Plk1 localization and spindle morphology. The result that U0126-treated oocytes could not properly localize Plk1 to the spindle poles and form the normal spindles provides us a probable explanation about how

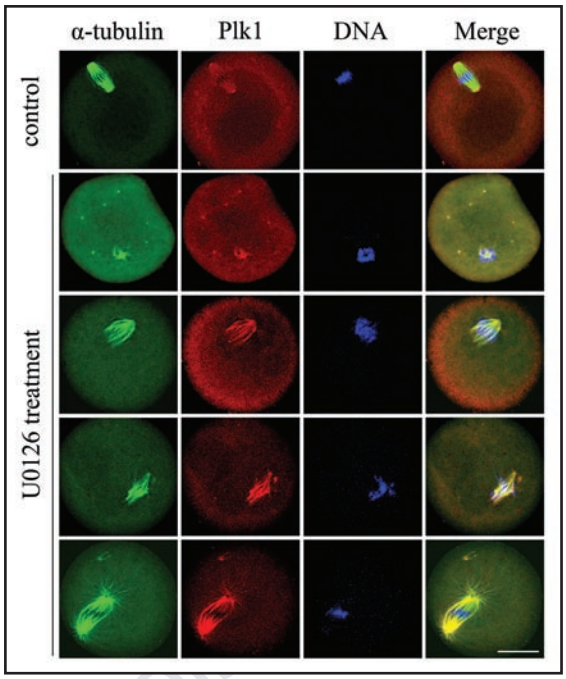

Figure 5. U0126 treatment leads to the abnormal localization of Plk1 and defective spindles in mouse oocytes. GV oocytes were cultured in the M16 medium containing 50 μM U0126 for 8 h and then double stained for Plk1 localization and spindle morphology. Green, α-tubulin; red, Plk1; blue, chromatin. Each sample was counterstained with Hoechst 33258 to visualize DNA. Scale bar, 20 μm.

spindle organization was affected in the absence of MEK1/2 activity. When MEK1/2 is blocked by U0126, Plk1 becomes dissociated from the spindle poles through some unknown mechanisms. Maybe the perturbed MEK1/2 could not phosphorylate and activate Plk1, which needs our further investigation. In this case, Plk1 fails to interact with or phosphorylate its spindle pole-localized substrates which are indispensable for the microtubule nucleation and bipolar spindle formation, such as Nlp (ninein-like protein), γ-tubulin and GTP-binding protein Ran, resulting in the severely impaired spindles in MEK1/2-inhibited oocytes.

In conclusion, there probably exists a multi-protein complex containing Plk1 and MEK1/2 in the regulation of meiotic spindle assembly, and Plk1 is involved in MEK1/2-regulated spindle formation during mouse oocyte meiosis.

Materials and Methods

Antibodies. Rabbit polyclonal anti-phospho-MEK1/2 (ser217/221) antibody was purchased from Cell Signaling Technology (Beverly, MA); mouse monoclonal anti-α-tubulin-FITC antibody and mouse monoclonal anti-Plk1 antibody were obtained from Sigma-Aldrich Co., (St Louis, MO).

Oocyte collection and culture. Animal care and use were conducted in accordance with the Animal Research Committee guidelines of the Institute of Zoology, Chinese Academy of Sciences.

Immature oocytes arrested at prophase I of meiosis were collected from ovaries of 6-week-old female Kunmin White mice in M2 medium (Sigma, St. Louis, MO). Only those immature oocytes displaying a germinal vesicle (GV) were cultured further in M16 medium under liquid paraffin oil at 37°C in an atmosphere of 5%

CO_2 in air. At different times after culture, oocytes were collected for immunostaining, drug treatment or coimmunoprecipitation.

Taxol and nocodazole treatment of oocytes. Oocytes at various stages were treated with taxol or nocodazole. For taxol treatment, 5 mM taxol (Sigma) in DMSO stock was diluted in M16 medium to give a final concentration of 10 μM and oocytes were incubated for 45 min; for nocodazole treatment, 10 mg/ml nocodazole in DMSO stock (Sigma) was diluted in M16 medium to give a final concentration of 20 μg/ml and oocytes were incubated for 10 min. After treatment, oocytes were washed thoroughly and used for immunofluorescence. Control oocytes were treated with the same concentration of DMSO in the medium before examination.

U0126 treatment of oocytes. For U0126 treatment, 10 mM U0126 in DMSO stock was diluted in M16 medium to give a final concentration of 50 μM. The GV oocytes were incubated in M16 medium containing 50 μM U0126 for metaphase I stage. Control oocytes were treated with the same concentration of DMSO in the medium before examination.

Immunofluorescence and confocal microscopy. For double staining of Plk1 and p-MEK1/2, oocytes were fixed in 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. After being permeabilized with 0.5% Triton X-100 at room temperature for 20 min, oocytes were blocked in 1% BSA-supplemented PBS for 1 h and incubated overnight at 4°C with 1:50 mouse anti-Plk1 antibody. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 for 5 min each, the oocytes were labeled with 1:100 FITC-conjugated goat-anti-mouse IgG for 1 h at room temperature. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100, the oocytes were again blocked in 1% BSA-supplemented PBS for 1 h at room temperature, then stained employing the method mentioned above except that the primary antibody was 1:100 rabbit anti-p-MEK1/2 antibody and the secondary antibody was 1:100 TRITC-conjugated goat-anti-rabbit IgG. Then the oocytes were further washed three times in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 and stained with Hoechst 33258 (10 μg/ml in PBS) for 20 min. Finally the oocytes were mounted on glass slides and examined with a Confocal Laser-Scanning Microscope (Zeiss LSM 510 META, Germany).

For double staining of Plk1 and α-tubulin, oocytes were fixed in 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. After being permeabilized with 0.5% Triton X-100 at room temperature for 20 min, oocytes were blocked in 1% BSA-supplemented PBS for 1 h and incubated overnight at 4°C with 1:50 mouse anti-Plk1 antibody. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 for 5 min each, the oocytes were labeled with 1:100 TRITC-conjugated goat-anti-mouse IgG for 1 h at room temperature. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100, the oocytes were again blocked in 1% BSA-supplemented PBS for 1 h at room temperature, followed by staining with 1:100 anti-α-tubulin-FITC antibody. Then the oocytes were further washed three times in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 and stained with Hoechst 33258 (10 μg/ml in PBS) for 20 min. Finally the oocytes were mounted on glass slides and examined with a Confocal Laser-Scanning Microscope (Zeiss LSM 510 META, Germany).

For triple staining of α-tubulin, Plk1 and p-MEK1/2, the same method mentioned above was employed, except that the secondary antibody for Plk1 was 1:100 Cy5-conjugated goat-anti-mouse IgG. Each experiment was repeated at least three times, and about 100 oocytes were examined in each group. The same instrument settings were used for each replicate.

Immunoprecipitation and immunoblotting analysis. Immunoprecipitation was carried out with rabbit polyclonal anti-p-MEK1/2 antibody according to the Instructions for ProFound Mammalian Co-Immunoprecipitation Kit (Pierce, Rockford, IL). Immunoblotting was then followed as previously described by us.[24] Plk1 was detected using 1:500 mouse monoclonal anti-Plk1 antibody. The membrane was processed using the enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ).

Acknowledgements

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