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***Short communication***

**Phosphorylated ERK5/BMK1 transiently accumulates within division spindles  
in mouse oocytes and preimplantation embryos**

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Running title: ERK5 in mouse oocytes and embryos

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**Abstract**

MAP kinases of ERK family play important roles in oocyte maturation, fertilization, and early embryo development. The role of the signaling pathway involving ERK5 MAP kinase during meiotic and mitotic M-phase of the cell cycle is not well known. Here we studied localization of phosphorylated, thus, activated form of ERK5 in mouse maturing oocytes and mitotically dividing early embryos. We show that phosphorylation/dephosphorylation, i.e. activation/inactivation of ERK5 correlates with M-phase progression. Phosphorylated form of ERK5 accumulates in division spindle of both meiotic and mitotic cells, and precisely co-localizes with spindle microtubules at metaphase. This localization changes drastically in anaphase when phospho-ERK5 completely disappears from microtubules and transits to the cytoplasmic granular, vesicle-like structures. In telophase oocytes it becomes incorporated into the midbody. Localization of phospho-ERK5 suggests that it may play important role both in meiotic and mitotic division.

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## Introduction

MAPK (Mitogen Activated Protein Kinase) signaling pathways are implicated in the regulation of wide range of cellular processes such as proliferation, differentiation, and apoptosis [1]. Their function has been analyzed in various somatic cells [2-6], as well as in oocytes and embryos (e.g. [7, 8]). BMK1/ERK5/MAPK7 (Mitogen-activated protein Kinase 1/Extracellular signal-Regulated Kinase 5/Mitogen Activated Protein Kinase 7; further called by us ERK5) is one of the less characterized MAP kinases of the ERK family. ERK5 mouse knock-out mice proved that the major developmental function of this kinase is related to the placental, cephalic, hindgut, and, importantly, cardiovascular development [9-13]. Thus, mouse embryos lacking functional ERK5 gene failed to progress beyond E10.5, but until this developmental stage the cellular proliferation seemed unperturbed. Further analyses suggested, however, that ERK5 is activated at G2-M transition, and thus, involved in the regulation mitotic division [2, 14-16]. Details of the mitotic function of this kinase have been analyzed in somatic cells, but have never been previously reported in oocytes or in embryonic cells.

Among the MAP kinases family members studied so far in oocytes and embryos, the function of only ERK1 and ERK2 is the best known. These two MAP kinases are activated by Mos-MEK1 pathway and are necessary for the establishment of CSF (cytostatic factor) mediated arrest of metaphase II oocytes in mouse [8, 17] as well as in frog [18]. Special attention was paid to ERK1 and ERK2 MAPK pathway since it was shown to be necessary to sustain the meiotic arrest of maturing vertebrate oocytes, establishment of oocyte polarity and impacting at the correct size of the polar body (e.g. [19-24]). In-gel MBP kinase assay performed using mouse oocytes and one-cell embryos proved that ERK1 and ERK2 activity is high exclusively in oocytes, but undetectable during mitotic divisions of early mouse embryos [8, 25]. The same assay showed also that other MBP (myelin basic protein, i.e. MAPK substrate) phosphorylating kinases are active both in oocytes and mitotically dividing embryos [8, 26], suggesting potential role of other MAP kinases in regulation of early embryonic mitosis. ERK5 is likely to fulfill this role. The evidence supporting the idea of ERK5 involvement both in meiosis and mitosis comes from analyses of MAP kinases substrates. Phosphorylated form of one of them, i.e. p90<sup>Rsk1</sup>, was detectable in meiotically dividing mouse oocytes, when ERK1 and ERK2 are fully activated, but also during the first mitosis of mouse embryo, when the two ERK kinases remain inactive [[25], our unpublished data]. Since p90<sup>Rsk1</sup> was shown to

be phosphorylated by ERK5 in somatic cells [3], it is likely that p90<sup>rsk1</sup> is activated by the same pathway during M-phase of oocytes and early embryos. ERK5-encoding mRNA was detected in unfertilized oocytes, and in preimplantation embryos up to blastocyst stage suggesting that the protein could be present at that stages [7]. The presence of ERK5 protein in oocytes was shown using Western blotting analysis of pig oocytes lacking precise localization analysis [27]. Similarly, Wang et al. presented Western blot analysis of preimplantation mouse embryos (3.5 days of development) and non-conclusive immunodetection of active and inactive forms of this antigens [7].

In the current paper we show that ERK5 is present in mouse oocytes and early embryo and that its activation is precisely regulated during each of M-phases studied and that its association with both meiotic and mitotic spindle is highly controlled during M-phase progression.

## **Material and Methods**

### *Ethical issues*

All animal studies presented were approved by Local Ethic Committee No. 1 in Warsaw, Poland according to European Union Council Directive 86/609/EEC of 24 November 1986. All animals were raised on the premises.

### *Collection and culture of oocytes at germinal vesicle stage*

Two- to three-month-old F<sub>1</sub> (C57Bl/10 x CBA/H) female mice were injected intraperitoneally with 10 IU pregnant mare serum gonadotrophin (PMSG; Folligon, Intervet, Netherlands) to stimulate the development of ovarian follicles. Forty-eight to fifty-two hours later females were killed by cervical dislocation. Fully grown oocytes arrested at prophase of the first meiotic division [germinal vesicle stage (GV)] were released from ovarian follicles. Oocytes were freed from cumulus cells by pipetting and then cultured for 2 h in M2 medium [medium M16 buffered with HEPES; [28]] containing bovine serum albumin (BSA; 4 mg/ml). Oocytes were fixed subjected to immunofluorescence at GV stage, at the GVBD (germinal vesicle breakdown) and at selected, subsequent stages of in vitro maturation.

*Collection, parthenogenetic activation of ovulated oocytes and in vitro culture of one- and two-cell embryos, blastocysts.*

F1 females were superovulated by injection of 10 IU of eCG and 10 IU of hCG (Intervet, Netherlands) 48-52 hours apart. Ovulated oocytes were collected 17-18 hours after hCG injection and cumulus cells were removed with hyaluronidase (Sigma Aldrich, Germany, 300 units/ml PBS). For the parthenogenetic activation the oocytes were exposed to 8% ethanol (POCh, Poland) in M2 medium for 8 minutes at room temperature [29], washed carefully and cultured in M2. Only those oocytes that extruded polar bodies and formed pronuclei were used for further analyses. Starting at 14 hours after activation embryos were scored every 10 minutes to determine the timing of the nuclear envelope breakdown (NEBD) and then collected for further analyses at different time after NEBD. Fertilized two-cell embryos were obtained from superovulated and mated females 49 hours after hCG. Embryos were collected and cultured in M2 medium. Starting from 49 hours after hCG embryos were observed every 10 minutes to determine the timing of NEBD and collected for further analyses at different times after NEBD. Blastocysts were obtained from uteri of superovulated and mated females at 90 hours after hCG.

#### *In vitro culture of murine NIH3T3 fibroblasts and C2C12 myoblasts.*

NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented (DMEM, Invitrogen) with 10 % fetal calf serum (FCS) and antibiotics (Invitrogen) at 37°C in 5 % CO<sub>2</sub>. Media were supplemented with penicillin (100 Units/ml) and streptomycin (100 mg/ml). Cells were fixed 48 hrs after plating and processed for Western blotting and immunodetection of tubulin and phospho-ERK5 epitopes. C2C12 murine myoblasts cell line was obtained from the European Collection of Cell Cultures (ECACC no. 91031101, passage no. 13). Cells were cultured in high-glucose DMEM supplemented with 10% FCS and antibiotics at 37°C in 5 % CO<sub>2</sub>. Cells were fixed and processed for immunodetection of tubulin and phospho-ERK5 epitopes at day 2 after plating, i.e. at the proliferative stage.

#### *Western blotting*

Protein samples of NIH3T3 cells were subjected to electrophoresis on 9 % SDS-PAGE gels [30]. Separated proteins were transferred to nitrocellulose membranes (Hybond C, Amersham Biosciences) and probed with rabbit polyclonal primary antibodies either against ERK5 or Phospho-ERK5 (Upstate) at the dilution of 1:1000. Next,  $\alpha$ -tubulin was immunodetected with mouse monoclonal antibody

(Sigma Aldrich, Germany). Antigen–antibody complexes were revealed using alkaline phosphatase conjugated anti-rabbit or anti-mouse secondary antibody (diluted 1:20,000, Jackson ImmunoResearch, USA) in combination with Enhanced Chemifluorescence reagent (ECF; Amersham Biosciences). Signal quantification was performed using ImageQuant 5.2 software (Amersham Biosciences).

### *Immunofluorescence*

NIH3T3 cells and C2C12 myoblasts plated and cultured on glass coverslips were fixed in 3.7 % paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1% Triton X100 in PBS for 5 min. Oocytes, embryos were fixed in 3.7 % paraformaldehyde in PBS for 30 min at room temperature and permeabilized with 0.1% Triton X100 in PBS for 5 min. Non-specific binding was blocked by 1 h long treatment with 3% BSA in PBS. Rabbit polyclonal antibodies against phospho-ERK5 (Upstate Biotechnology, USA) were used at the dilution of 1:200 and the incubation was performed overnight at 4°C. Next,  $\alpha$ -tubulin was immunodetected with mouse monoclonal antibody (Sigma Aldrich, Germany) followed by FITC-labeled anti-rabbit and TRITC-labeled anti-mouse antibodies (Jackson ImmunoResearch, USA). Chromatin was visualized either with chromomycin A3 (Sigma Aldrich, Germany) diluted 1:1000 in PBS with 5 mM MgCl<sub>2</sub> for 0.5 h at room temperature or propidium iodide present in mounting medium (Vector Laboratories, USA). Samples incubated in chromomycin A3 were mounted in Citifluor (Citifluor Ltd, Great Britain) on glass slides. Immunofluorescence analysis was accompanied by appropriate negative controls, i.e. samples that were not subjected to the primary antibodies, but instead they were incubated in 3% BSA in PBS. All samples were examined with laser scanning confocal microscope (Axiovert 100M, Carl Zeiss Jena, Germany) and processed with Adobe Photoshop 6.0.

## **Results & Discussion**

Since ERK5 is known to be phosphorylated upon M-phase in human cells [2, 16, 31], and its role in mammalian meiosis is virtually unknown, we decided to begin characterization of this MAP kinase in mouse oocytes by follow the dynamics in its phosphorylation state using a specific anti-phospho-ERK5 (P-ERK5) antibody.

In germinal vesicle (GV) stage oocytes (meiotic prophase I) phosphorylated ERK5 is absent from the GV and is detected as a background staining within the

cytoplasm (Fig. 1, GV). Following M-phase entry, upon germinal vesicle breakdown (GVBD), the P-ERK5 signal becomes detectable close to condensing bivalents (Fig. 1, GVBD + 1h). This staining increases with the progression of the prometaphase and reflects organization of microtubules forming meiotic spindle around the individualized bivalents. The intensity of the staining is the highest in the vicinity of the chromosome bivalents (Fig. 1, GVBD+3h). In metaphase of the first meiotic division P-ERK5 localizes entirely to the spindle microtubules and the background, i.e. cytoplasmic staining, becomes negligible (Fig. 1, metaphase I). At the time of the first polar body extrusion, at the anaphase I/telophase I transition, P-ERK5 is no more detectable on the spindle microtubules, however, remains clearly positioned next to telophase groups of chromatin and within the midbody, concomitantly with the decrease in the cytoplasmic staining (Fig. 1, telophase I). During the metaphase of the second meiotic division P-ERK5 staining becomes again localized exclusively to the meiotic spindle, as during the metaphase I (Fig. 1, metaphase II). At this stage the cytoplasmic staining is also reduced to the minimum. Thus, the P-ERK5 localization in oocytes undergoing meiotic maturation shows that active form of ERK5 MAP kinase localizes specifically to the meiotic spindle. The affinity of ERK5 to the spindle microtubules manifests very early upon GVBD and progresses during the long, taking about 8 hours, metaphase I. The progressive incorporation of ERK5 to the spindle is manifested by its total disappearance from the cytoplasm when the meiotic spindle is fully assembled both in metaphase I and II. Microtubule-associated localization of other MAP kinases, and some of their activators was reported before. ERK1/ERK2 activating MEK1/2 kinase was shown to be limited to the meiotic spindle, and its active form was shown to be located within the midbody during anaphase [32, 33]. In contrary ERK1/2 MAP kinases were detectable at the spindle poles [8]. ERK3 was also recently reported to co-localize to spindle microtubules, however, the activation of this kinase was not investigated [34]. The localization and activation of ERK5 in mouse oocytes and early embryos was not precisely studied so far, however, its expression was shown in pig oocytes [27], and mouse preimplantation embryos [7]. However, images of ERK5 and P-ERK5 localization in mouse blastocysts, presented by Wang and colleagues showed high, and uniform signal within the cytoplasm of inner cell mass and trophectoderm cells [7], clearly different that in oocytes studied by us. This prompted us to analyze the P-ERK5 localization also in preimplantation mouse embryos.



To verify the specificity of the antibody used we performed Western blotting and immunolocalization analyses of total ERK5 and P-ERK5 in murine somatic NIH3T3 cells. Similarly as presented by others (e.g. [2, 16]) we detected a clear increase in phosphorylated, up-shifted form of ERK5 in nocodazole-synchronized, i.e. mitotic cells. These result was obtained using both anti-total ERK5 (Fig. 2A, ERK5) and anti-P-ERK5 antibody (Fig. 2B, P-ERK5). Importantly, the phosphorylated form of ERK5 localized not only to the meiotic spindles, as described above, but also to the mitotic spindle, as shown for mouse NIH3T3 cells (Fig. 2B) and in murine C2C12 myoblasts (Fig. 2C). In the interphase NIH3T3 and C2C12 cells a low cytoplasmic staining is detectable, while the nuclei are entirely devoid of P-ERK5 (Fig. 2B and 2C, white asterisks). The nuclear localization of P-ERK5 becomes clear during prophase (Fig. 2B and C, prophase). Further, in M-phase, the P-ERK5 staining is restricted to the mitotic spindle only in NIH3T3 cells (Fig. 2B, M-phase), while in murine myoblasts C2C12 P-ERK5 colocalizes not only to the spindle, but also to chromosomes (Fig. 2C, M-phase). Thus, Western blotting and also immunolocalization confirm that the signal detected in murine oocytes corresponds to the phosphorylated form of ERK5.

To perform precise analysis of P-ERK5 during M-phase progression in embryonic cells, we studied the well characterized model of mitotically dividing mouse one-cell embryos. The first mitotic division of mouse embryo can serve, due to its length of approximately 120 minutes, as a useful tool to study the mitotic events [35-39]. This feature allowed us to precisely analyze the localization, and dynamics of ERK5 activation in M-phase. At metaphase of the first mitotic division of one-cell embryos, similarly as it was shown for oocytes, P-ERK5 perfectly colocalized to the spindle microtubules (Fig. 3A, metaphase). Moreover, the cytoplasmic staining was not detected. As soon as the anaphase started the pattern of P-ERK5 localization changed dramatically. First of all the P-ERK5 signal was lost from the microtubules, and becomes visible as diffused cloud around the spindle (Fig. 3A, early anaphase). This signal dispersed during the progresses to late anaphase and beginning of cytokinesis (Fig. 3A, late anaphase). Concomitantly, the cytoplasmic staining increased (Fig. 3A, early and late anaphase). Thus, following the metaphase-anaphase transition P-ERK5 is no longer present on spindle microtubules, but seems to disperse around the anaphase spindle and further within the cytoplasm. Similar localization of P-ERK5 within the mitotic spindle was observed in two cell stage

blastomeres (Fig. 3B), and at further stages of preimplantation development (Fig. 3C).

These results show that: 1) ERK5 is phosphorylated upon M-phase entry in meiosis and mitosis; 2) in both cases it localizes to the metaphase spindle microtubules; 3) and disappears from the spindle in early anaphase. Such localization suggests that ERK5 plays a similar function in spindle assembly/function in oocytes, embryos and somatic cells.

ERK5/ BMK1/MAPK7 belongs to the subfamily of the MAP kinase family in mammals, and it has some characteristic features: a large C-terminus and a unique loop 12 sequence [40]. Mammalian ERK5 was shown to be activated by oxidative stress, hyperosmolarity and several growth factors, including epidermal growth factor and nerve growth factor [40-45]. The mitotic function of ERK5 was suggested by the results of the experiments focusing at both non-transformed cells such as fibroblasts and transformed cell lines such as HeLa cells. In HeLa cells expressing of constitutively active MEK5, and as a result activation of ERK5, leads to the increase in the number of mitotic cells [2]. On the other hand the use of the dominant negative form of ERK5, or siRNA suppressing ERK5 expression reduced mitotic index of analyzed cells [2]. Moreover, siRNA depletion of ERK5 decreases cell survival of mitotically dividing cells [14]. However, the fact that mouse embryos lacking functional ERK5 gene are able to survive until approximately E10.5 strongly suggests that ERK5 function can be replaced by another enzyme. Other MAP kinases, like ERK1 and ERK2, are candidates for such function. Despite that localization of ERK1 and ERK2 does not precisely reflect the P-ERK5 position within the mitotic spindle, it is well known that these kinases play a crucial function in meiotic spindle organization and function [8]. Analyses of oocytes of Mos kinase, an upstream ERK1/2 activating kinase, deficient mice, proved that ERK1/2 regulate microtubule dynamics [17, 19, 20]. Recent discoveries of ERK3 localization and function in mouse oocytes identify a new candidate, which together with ERK1/2 and ERK5, might be involved in the regulation of spindle dynamics [34]. ERK3 was shown to be crucial for the successful mouse development, but again, similarly as it was described for ERK5 knock-out mice, ERK3 deficiency did not prohibit mitotic divisions of vast majority of embryonic tissues [46]. This kinase, however, similarly do ERK1/2 and ERK5, was shown to be phosphorylated during M-phase entry and dephosphorylated at metaphase/anaphase transition [47]. The ERK4-deficient mice also did not reveal any significant mitotic

phenotype, moreover, these animals were not only viable but also fertile [48]. Thus, only the perturbation of the activity ERK1/2 MAP kinases pathway, as shown by Mos knock-out mice analyses, has fatal consequences for the mouse fertility impacting at the oocyte maturation [49, 50]. Mos knockout does not influence the mouse embryo development and animal viability probably due to ERK1 and ERK2 activation via Ras/Raf pathway [51]. However, it is possible that other MAPK, such as ERK5, may also act to rescue Mos deficiency following fertilization. Further studies will be necessary to solve this issue.

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## Legend to Figures

### Fig. 1.

**P-ERK5 localization in maturing mouse oocytes.** Oocytes were analyzed at GV stage, and then during the formation of the metaphase plate and spindle of the first meiotic division (1 and 3 h after GVBD), at metaphase I (5 h after GVBD), telophase I (8 h after GVBD) and at metaphase II (9 h after GVBD). Top row - immunolocalization of P-ERK (green) and chromatin visualization (blue), overlay; bottom row - immunolocalization of P-ERK (green).

### Fig. 2.

**P-ERK5 in murine NIH3T3 fibroblasts and C2C12 myoblasts.** **A.** Immunoblotting of ERK5 and P-ERK5 in lysates of asynchronously growing and nocodazole synchronized NIH3T3 fibroblasts. Note the presence of upper, slow-migrating, i.e. phosphorylated form of ERK5 in nocodazole treated cells. Immunodetection of  $\alpha$ -tubulin was performed to show the equal loading of the analyzed samples; **B.** Immunolocalization of P-ERK5 in asynchronously growing NIH3T3 fibroblasts' **C.** Immunolocalization of P-ERK5 in asynchronously growing C2C12 myoblasts. NIH3T3 and C2C12 cells were fixed in interphase, prophase and metaphase. Top row – immunolocalization of P-ERK5 (green),  $\alpha$ -tubulin (red), and visualization of chromatin (blue), overlay; middle row - immunolocalization of  $\alpha$ -tubulin (red); bottom row - immunolocalization of P-ERK5 (green). Note the P-ERK accumulation in prophase nuclei and spindle and chromosome localization in metaphase. White stars indicate interphase nuclei lacking P-ERK5 staining.

### Fig. 3

**P-ERK5 localization in preimplantation mouse embryos.** **A.** One-cell mouse parthenogenetic embryos fixed at metaphase and anaphase of first mitotic division. **B.** Two-cell mouse embryo fixed at metaphase of the second mitotic division. **C.** Mitotically dividing inner cell mass in blastocyst. Top row – immunolocalization of P-ERK5 (green),  $\alpha$ -tubulin (red), and visualization of chromatin (blue), overlay; middle row - immunolocalization of  $\alpha$ -tubulin (red); bottom row - immunolocalization of P-ERK5 (green).

Fig. 1

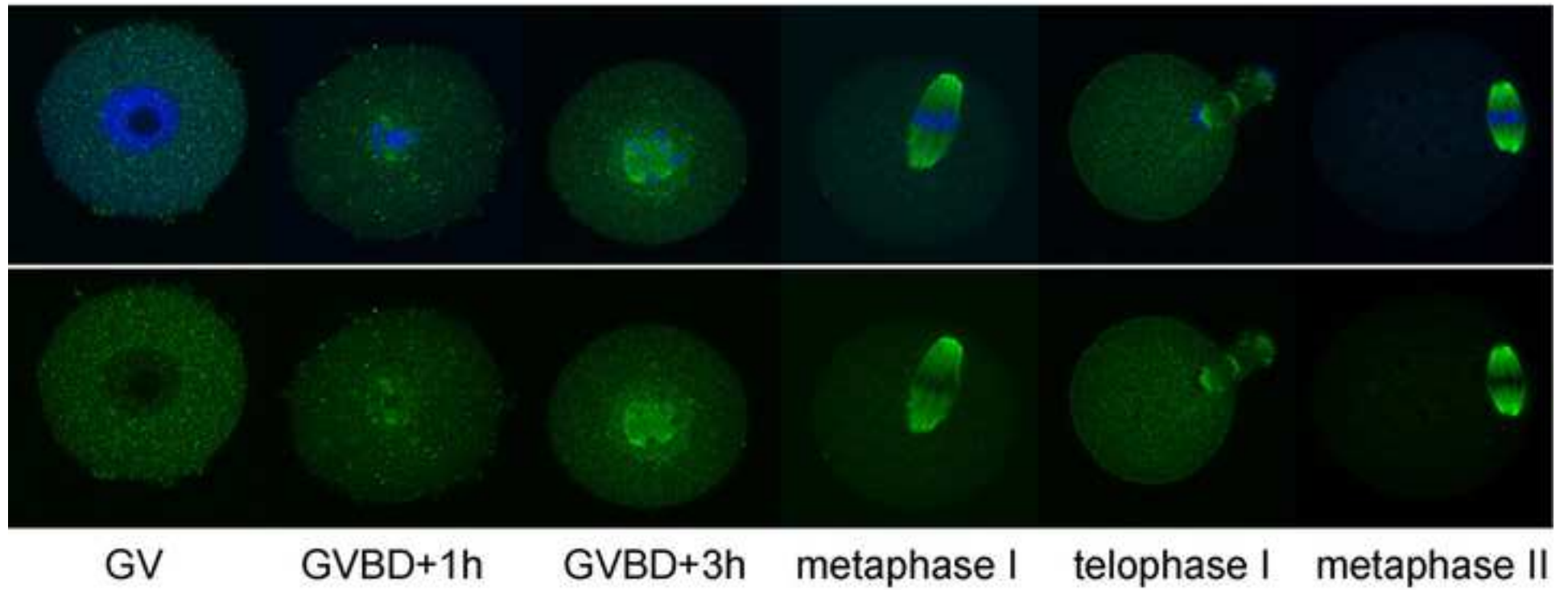




Fig. 2

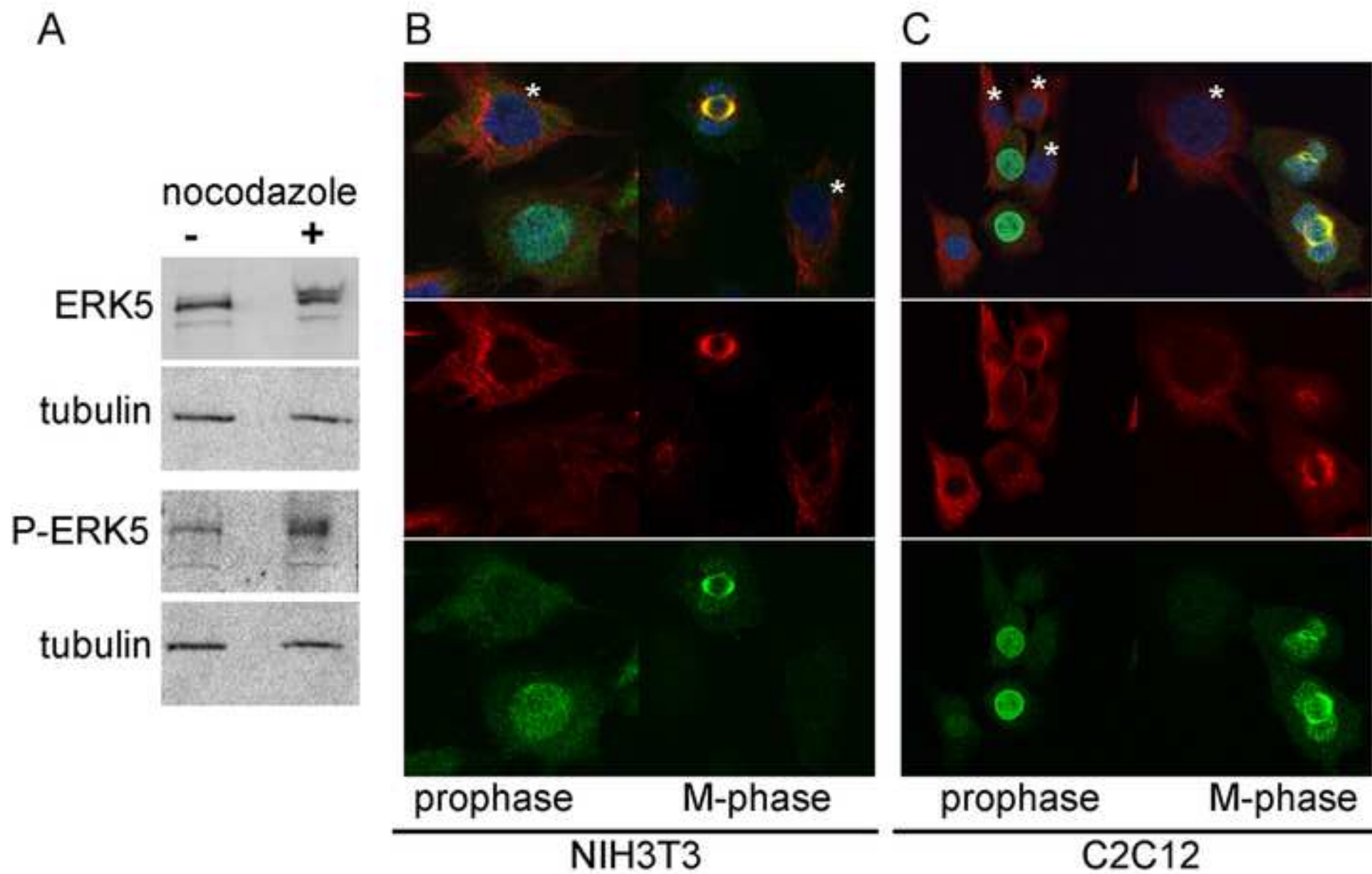


Fig. 3

