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MAPK/ERK activity is required for the successful progression of mitosis in sea urchin embryos

Odile Mulner-Lorillon[a,b,*], Héloïse Chassé[a,b], Julia Morales[a,b], Robert Bellé[a,b], Patrick Cormier[a,b]

[a] Sorbonne Universités, UPMC Univ Paris 06, UMR 8227, Integrative Biology of Marine Models, Translation Cell Cycle and Development, Station Biologique de Roscoff, CS90074, F-29688 Roscoff cedex, France

[b] CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, CS90074, F-29688 Roscoff cedex, France

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ABSTRACT

Using sea urchin embryos, we demonstrate that the MEK/MAPK/ERK cascade is essential for the proper progression of the cell cycle. Activation of a limited fraction of MAPK/ERK is required between S-phase and M-phase. Neither DNA replication nor CDK1 activation are impacted by the inhibition of this small active MAPK/ERK fraction. Nonetheless, the chromatin and spindle organisations are profoundly altered. Early morphological disorders induced by the absence of MAPK/ERK activation are correlated with an important inhibition of global protein synthesis and modification in the cyclin B accumulation profile. After appearance of morphological disorders, there is an increase in the level of the inhibitor of protein synthesis, 4E-BP, and, ultimately, an activation of the spindle checkpoint. Altogether, our results suggest that MAPK/ERK activity is required for the synthesis of (a) protein(s) implicated in an early step of chromatin /microtubule attachment. If this MAPK/ERK-dependent step is not achieved, the cell activates a new checkpoint mechanism, involving the reappearance of 4E-BP that maintains a low level of protein translation, thus saving cellular energy.

1. Introduction

Mitogen-activated protein kinases (MAPKs) are members of signal transduction pathways, conserved from yeast to humans, which convert extracellular stimuli into a range of cellular responses. Among the seven well-characterized MAPKs modules, the canonical MAPK/ERK pathway was shown to play a central role in cell proliferation control (reviewed in Cargnello and Roux (2011)). The MAPK/ERK module is composed of a set of three sequentially acting kinases: the c-Raf (cellular proto-oncogene of Rapidly Accelerated Fibrosarcoma gene) family members Raf-1/A/B, which phosphorylate and activate the MEK1/2 (MAPK/ERK Kinases), which in turn phosphorylate and activate the ERK1/2 (Extracellular signal-Regulated Kinases). In most cells mitogenic agents, growth factors or insulin stimulate the MAPK/ERK pathway through cell surface receptors, such as receptor tyrosine kinases (RTKs). Raf is activated by phosphorylation and/or interaction with small GTP-binding proteins of the Ras/Rho family. Upon stimulation, MAPK/ERK phosphorylates a large number of substrates, transcription factors (Elk1, c-fos, c-myc,...), membrane, cytoskeletal or chromatin-associated proteins (calnexin, MAP4, CENP-E,...), as well as signalling proteins (TSC2, PLC,...) (Yoon and Seger, 2006). Additionally, MAPK/ERK phosphorylates and activates other kinases, mainly RSK1/4 (p90 ribosomal S6 *kinases; p90^{RSK}*) and MNK1/2 (MAPK-interacting *kinases), which, through their own substrates, further expand the biological processes regulated by the MAPK/ERK pathway*. MAPK/ERK, RSK, MNK phosphorylate and regulate a number of components of the translational machinery (rpS6, eIF4B, eIF4E, eIF4G, eEF2K), as well as some mRNA-binding proteins and splicing factors (reviewed in Cargnello and Roux (2011)). Thus, the MAPK/ERK pathway participates in the control of global and selective protein synthesis. Furthermore, phosphorylation of TSC2 by MAPK/ERK and RSK and of raptor by RSK is reported to regulate the mTOR pathway, the master regulator of translation initiation (Cargnello et al., 2015).

Normal progression through the various phases of cell cycle is governed by CDKs, a family of kinases who's activity depends on their phosphorylation status, on the synthesis/degradation of their cyclin partners and the presence of specific inhibitors (reviewed in Nigg (2001)). Fine-tuned regulation of cell cycle is of crucial importance to avoid cellular transformation. The CDKs are under control of multiple checkpoints, acting through different kinase cascades to ensure genomic integrity and cell survival (reviewed in Malumbres (2014)). Thus, sustained MAPK/ERK activation was reported to regulate efficient G1/S transition. MAPK/ERK, directly or via RSK and MSK, phosphorylates

* Corresponding author at: Station Biologique de Roscoff, CS90074, F-29688 Roscoff cedex, France.
E-mail address: mulner@sb-roscoff.fr (O. Mulner-Lorillon).

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and (i) activates several transcription factors involved in the expression of cell cycle regulators (cyclin D, cdk4, cdc25, p21…), and (ii) inhibits $p27^{kip1}$ and $p21^{cip1}$, which are potent G1-CDKs inhibitors (reviewed in Cargnello and Roux (2011)). On the other hand, MAPK/ERK signalling is generally considered as dispensable for mitosis to proceed (Meloche and Pouyssegur, 2007), nevertheless it has been reported to exert a modulatory role in early G2 for timely progression (Rieder, 2011).

Evidence for a regulatory role of the MAPK/ERK pathway on S/G2/M phases of the cell cycle come from studies on meiotic maturation in metazoan oocytes, primarily *Xenopus* and starfish. Meiotic maturation is triggered by the universal M-phase factor, initially known as MPF and identified as the CDK1/cyclin B complex. Meiotic maturation occurs at the time of ovulation when full-grown oocytes, arrested in first meiotic prophase, are induced to resume meiosis by going through the first and the second meiotic division, arresting again until fertilization, most often in meiotic metaphase II or in G1 phase. It is thus considered to be a model for a G2/M transition (reviewed in Kishimoto (2003)). The MAPK/ERK pathway was shown to contribute to the amplification of MPF activation through the RSK-mediated phosphorylation and (i) inhibition of Myt1 (CDK1 inhibitory kinase) and (ii) activation of CDC25 (CDK1 activating phosphatase) (reviewed in Dupre et al. (2011) and Kishimoto (2003)). In addition, the MAPK/ERK pathway was characterized as the key component of the cytostatic factor (CSF) that is ubiquitously responsible for the cell cycle arrest of matured oocytes before fertilization (reviewed in Costache et al. (2014)). In *Xenopus*, the MII-arrest depends on the inhibition of the anaphase-promoting complex (APC), induced by the RSK-dependent phosphorylation of two inhibitors of APC, Bub1 and Emi2/Erp1 (Dupre et al., 2011). In starfish, the Mos/MAPK pathway was reported to arrest cell cycle by a dual–lock involving (i) the RSK-dependent inhibition loading on chromatin of cdc45, a component of the replication complex (Tachibana et al., 2010) and (ii) the RSK-independent suppression of M-phase cyclin synthesis (Hara et al., 2009).

Although these studies give valuable insights about the mechanism of MAPK/ERK control of the G2/M phases of the cell cycle, it must be emphasized that the control would be by some means different in a mitotic cell cycle. In most cells, the MAPK/ERK pathway is activated in response to a variety of extracellular growth-factor/receptor interactions and leads to transcriptional activation that influences a number of tissue specific biological activities, cell proliferation, survival and differentiation. In contrast, in oocytes MAPK/ERK is activated independently of growth factors and tyrosine receptors, by the regulation of the level of a germ cell specific MEK-activating kinase: the c-mos proto-oncogene product (Dupre et al., 2011).

Sea urchin eggs are recognized as a powerful model to study molecular mechanisms of S/G2/M phase regulation, in the context of naturally synchronized cells entering cell cycle in response to a physiological stimulus, *i.e.* fertilization. Sea urchin eggs that have completed their meiotic maturation are haploid cells blocked in G1. Fertilization triggers entry into the early synchronous embryonic cell division, along with a dramatic rise in protein synthesis that is independent of mRNA transcription. *De novo* protein synthesis is dispensable for S-phase progression but is required for the onset and normal progression of mitosis (reviewed in Epel, 1990). Among the proteins required to enter mitosis is cyclin B, the regulatory subunit of universal M-phase factor, the CDK1/cyclin B complex. We previously reported that cyclin B synthesis after fertilization mainly depends on a mTOR-sensitive pathway, which triggers the release of the translation inhibitor 4E-BP from eIF4E (eukaryotic Initiation Factor 4E) and the degradation of 4E-BP (Cormier et al., 2003, 2016; Chasse et al., 2016). A requirement for a MAPK/ERK regulatory pathway at the time of fertilization in sea urchin was essentially investigated at the level of the G1 arrest in unfertilized sea urchin eggs. It was thus reported that the cell cycle arrest in sea urchin relies on maintenance of high MAPK/ERK activity and that fertilization triggers a rapid and drastic MAPK/ERK inactivation responsible for entry into S-phase (Pelech et al., 1988; Chiri et al., 1998; Carroll et al., 2000; Kumano et al., 2001; Zhang et al., 2005, 2006). Instead, a possible involvement of the MAPK/ERK pathway after this step has been the subject of contradictory reports (Chiri et al., 1998; Pesando et al., 1999; Zhang et al., 2005). We therefore decided to investigate the implication of the MAPK/ERK pathway during the mitotic cell division after fertilization in sea urchin embryos.

Using the specific MEK kinase inhibitor U0126 (Bain et al., 2007), we demonstrate that a low level of MAPK phosphorylation appears to be needed between S- and M-phase to allow proper cell cycle progression. We further show that this MAPK/ERK activity interferes neither with DNA replication nor with CDK1/cyclin B activation, indicating that the MAPK/ERK target is not involved in a classical G2/M replication checkpoint. Activated MAPK is needed for the stimulation of protein synthesis, as well as for normal cyclin B accumulation. Inhibition of MAPK/ERK induces chromatin/microtubule anomalies and triggers the accumulation of the translation inhibitor 4E-BP. Our results suggest that MAPK/ERK is involved in the synthesis of (a) protein(s) required to ensure the early attachment of chromatin to microtubules. This MAPK/ERK dependent step would be controlled by a checkpoint which, when stimulated, maintains a low protein synthesis activity to spare cellular energy.

2. Material and methods

2.1. Chemicals

The MEK inhibitor, U0126 was purchased from InvivoGen. [^{35}S] L-methionine (10 mCi/mmol) and [methyl-^{3}H] thymidine (70–90 Ci/mmol) were purchased from Perkin–Elmer. Rabbit polyclonal antibodies directed against *S. granularis* cyclin B (Lozano et al., 1998) were a generous gift from Professor Gérard Peaucellier (Banyuls, France). Rabbit polyclonal antibodies directed against *S. granularis* 4E-BP were previously described (Oulhen et al., 2010). Aphidicolin, mouse monoclonal antibodies directed against human PSTAIR (P7962) and against activated (Diphosphorylated ERK-1 & 2) MAPK (P-MAPK/ERK) (M8159) and rabbit polyclonal antibodies against human (ERK-1, ERK-2) MAPK (MAPK/ERK) (M5670) were purchased from Sigma Aldrich. Rabbit monoclonal antibodies directed against human (phospho^{320}threonine) catalytic subunit alpha of protein phosphatase 1 (P-PP1Cα) (ab62334) were obtained from Abcam. Alexa Fluor 488-conjugated mouse monoclonal alpha-Tubulin antibodies were from Invitrogen. Peroxydase-conjugated secondary antibodies were obtained from Dako. Amersham ECL western blotting detection reagents were from GE-Healthcare and ECL2 western blotting substrate was from Pierce.

2.2. Handling of gametes and embryos

Sphaerechinus granularis collected in the Brest area (France), were obtained from CRBM (Centre de Ressources Biologiques Marines) at the Roscoff Biological Station. Spawning of gametes was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in 0.22 µm Millipore-filtered seawater (FSW) and rinsed twice by centrifugation (2000 rpm, 2 min). Eggs were de-jellied by swirling twenty seconds in 0.7 mM citric acid pH5 and rinsed three times with fresh FSW. For fertilization, eggs were suspended in FSW (5% v/v suspension). Diluted sperm was added to the eggs. Experiments were only conducted with batches exhibiting more than 90% fertilization; in each experiment gametes from a single animal were used. Cultures were performed at 16 °C under constant agitation. At the indicated time, U0126 was added at the indicated final concentration to the egg suspension from a 20 mM stock DMSO solution.

2.3. Determination of cleavage rates and cytological analysis

Cleavage was scored at regular time intervals during 3 h after fertilization by observation under a phase contrast microscope. Thousands of embryos were incubated for each experimental determination, from which around 100 were scored for the occurrence of cleavage. Cytological analyses were performed on 0.2 ml aliquots of embryo suspension taken at various times after fertilization. After overnight fixation of the cells in 1 ml methanol/glycerol (3:1, v/v), in the presence of the DNA dye Hoechst 33258 (bisbenzimide, 0.1 µg/ml) the fixed cells were suspended in a 50% glycerol solution and mounted onto slides for microscope observation The nuclear envelope and DNA were observed under Nomarski differential interference contrast (DIC) and Zeiss fluorescence microscopy, respectively. For microtubule analysis, embryos taken at the different indicated times after fertilization were extracted for 1 h at 4 °C with 25 mM MES pH 6.8; 10 mM EGTA; 0.55 mM MgCl2; 25% glycerol; 1% NP40; 1 mM AEBSF and then fixed overnight at 4 °C in cold 90% methanol and 50 mM EGTA. After rehydratation in 0.05% Tween in PBS, followed by 1 h saturation in the presence of 1% BSA, eggs were incubated overnight at 4 °C in Alexa Fluor-conjugated alpha-tubulin antibody (1/50^{e}). DNA staining was performed using the DNA dye Hoechst 33258 (1 µg/ml in PBS) applied during the last rinse after incubation with the antibodies.

2.4. Embryo extracts and Western blot analyses

At different times following fertilization, total extracts were obtained by direct solubilization of 20 µl pelleted cells (eggs or embryos) in 150 µl of SDS-Fix buffer containing 2% sodium dodecyl-sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris HCl pH 6.8.

Proteins were resolved by SDS-PAGE (Laemmli, 1970). 4E-BP levels were assessed after sample resolution on a gel containing 15% acrylamide/bisacrylamide from a 40% stock solution (acrylamide/bis acrylamide; 2.6% C), and P-PP1Cα, P-MAPK/ERK and cyclin B analyses were performed on a 12% acrylamide gel containing 0.1% bisacrylamide.

Western blot analyses were performed following electrophoretic transfer of proteins from SDS-PAGE onto 0.22 µm nitrocellulose membranes (Towbin et al., 1979). Membranes were incubated with antibodies against cyclin B (1:1000), 4E-BP (1:5000), P-PP1Cα (1:1000), PSTAIR (1:1000), P-MAPK/ERK (1:1000) and MAPK/ERK (1:1000). The antigen-antibody complex was measured by chemiluminescence using horseradish peroxidase-coupled secondary antibodies according to the manufacturer's instructions (ECL or ECL2). The signals were quantified using ImageJ software.

2.5. Analysis of CDK1/cyclin B activation in vivo

The activation state of CDK1/cyclin B was determined by monitoring the endogenous ^{320}Threonine-phosphorylation status of PP1Cα, the catalytic subunit of protein phosphatase1 that was shown to be a natural substrate for CDK1 (Lewis et al., 2013). Measurements were done by western blotting after electrophoretic resolution of total protein extracts from embryos taken at various time after fertilization.

2.6. DNA synthesis in vivo

Ten minutes before fertilization, [methyl-^{3}H] thymidine (10µCi/ml) was added to a 5% egg suspension. When used, U0126 (60 µM) or aphidicholin (10 µg/ml) were added 5 min post-fertilization. At different times following fertilization, embryos from 1 ml samples of the suspension were rapidly packed by centrifugation for 5 s in an Eppendorf centrifuge, rinsed in Millipore-filtered seawater, and suspended in 1 ml ice-cold 20% trichloroacetic acid (TCA). Aliquots (50 µl) were taken for the determination of total thymidine uptake by counting in the presence of Optiphase Supermix scintillation liquid. After precipitation for one night at 4 °C, the pellets were collected by centrifugation, then washed twice with 20% TCA. The pellets were dissolved in 0.125 M NaOH (1 ml). Radioactivity was measured on 500 µl duplicate aliquots of the dissolved pellets, in the presence of Optiphase Supermix scintillation liquid.

2.7. Protein synthesis in vivo

Eggs (5% suspension in FSW) were fertilized in the presence or absence of 60 µM U0126. Every 15 min, batches of embryos (500 µl of the 5% suspension) were pulse-labelled for 10 min with 10 µCi/ml [^{35}S]-L-Methionine. After rinsing in FSW, cells were pelleted and frozen in liquid nitrogen. Cell extracts were prepared by re-suspending the pellets in 400 µl ice-cold buffer (40 mM HEPES pH7.6, 100 mM NaCl, 0.4 mM EDTA, 2 mM DTT, 0.2 mM sodium orthovanadate, 20 mM PPi, 100 mM sodium fluoride, 100 mM β-glycerophosphate, and 10 µg/ml Protease Inhibitor Cocktail (P2714, Sigma Aldrich). Total [^{35}S]-methionine uptake was determined by counting the radioactivity present in duplicate aliquots of the extract. [^{35}S]-methionine incorporation into proteins was measured on duplicate aliquots after 10% TCA precipitation on Whatman 3 M filters and by counting in the presence of Optiphase Supermix scintillation liquid. The results, expressed in arbitrary units, correspond to the percentage of [^{35}S]-methionine incorporation into proteins over total radioactivity recovered in the cells. An aliquot of each supernatant was loaded on 12% acrylamide SDS-PAGE gel resolved. Radioactive proteins were visualized by autoradiography of the gels on Kodak Biomax MR films.

3. Results

3.1. U0126 and embryonic cell divisions

The effect of U0126 on the kinetic of cell division was analysed in *Sphaerechinus granularis* embryos following fertilization. The eggs were fertilized in the presence of different U0126 concentrations, cultured in drug-containing medium, and scored for the first embryonic division during 3 h post-fertilization. U0126 affected, in a dose-dependent manner, the occurrence of cell division, as judged by the percentage of 2-cell embryos observable in the presence of the drug (Fig. 1A). At 20 µM the drug induced a variable delay in the time of fertilization-induced division, whereas at 60 µM it completely and consistently prevented the division of the embryos. In experiments performed with gametes from five independent sets of parents, it was determined to have an ED50 of 35.0 µM (SEM 2.3), which fits with the dose recommended in the broad-spectrum study of the specificity of protein kinase inhibitors (Bain et al., 2007).

The effect of U0126 was not lethal and did not provoke irreversible early developmental damage since (i) when 60 µM treated embryos were washed free from the drug, they readily proceeded to cell division and (ii) embryos treated with 40 µM U0126, and which presented a large delay for the first cell division, remained healthy and further developed to at least the morula stage.

The window of time for U0126 action was examined by scoring cell division after U0126 addition (60 µM) to the incubation medium of the embryos at different times after fertilization. Fig. 1B shows the mean value of the results obtained for embryos from 5 different sets of parents. When the drug was added at 0 or 30 min post-fertilization, the first cleavage was severely impaired or totally prevented for more than 3 h. Added at 60 min, U0126 induced a significant delay in the occurrence of cell division, and 20–40% of the embryos did not reach the first cell division, 3 h after fertilization. When applied at 90 or 120 min post-fertilization, the drug had no effect on the first division. Noteworthy, in these cases, the second division was blocked or severely delayed, and embryos never reached the morula stage (data not shown). This result indicates that the drug has an effect not only at the level of the first division following fertilization, but is also effective

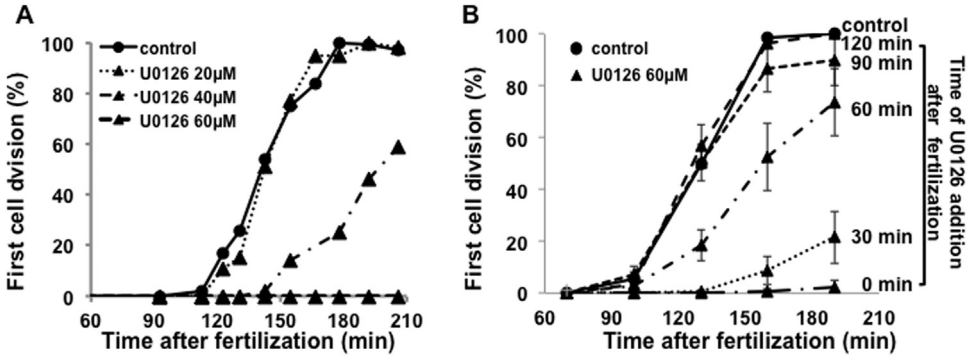

Fig. 1. Effect of U0126 on the first cell division after fertilization. (A) Dose-response effect of U0126 on the first mitotic division of sea urchin early development. Batches of *S. granularis* eggs (5% cells/vol solution in FSW) were incubated immediately after fertilization in the presence of 20 μM, 40 μM or 60 μM U0126. Cleavage rates were scored at different times during the time culture in the continuous presence of the drug. The curve was obtained from embryos isolated from a single set of parents and is representative of 5 independent experiments. (B) U0126 (60 μM) was added to batches of embryos at different times (0–120 min) after fertilization. Embryos were then kept in the presence of the drug and scored for cleavage rates. The curves represent the mean (+/- SEM) of the curves obtained in 5 different experiments, performed on batches of embryos from a single pair of parents.

at the second mitotic cell cycle suggesting a general role for the inhibitor target at each cell cycle.

Altogether these experiments demonstrate that the critical time for the effect of U0126 on entry into mitosis occurs during the first 60 min following fertilization, approximately 1 h before cell division.

3.2. U0126 and MAPK/ERK activation in sea urchin eggs

As shown above, the effect of U0126 in sea urchin occurs in a narrow concentration window (between 20 and 60 μM), characteristic of the existence of a switch-like, ultrasensitive response. Such responses are usually generated for the effective transmission of signals along a signalling cascade (Ferrell and Ha, 2014). The universal target for U0126 is MEK1, the MAPK/ERK phosphorylating and activating kinase in the MAPK module (Cargnello and Roux, 2011). We therefore followed the effect of U0126 on the phosphorylated-MAPK/ERK level by western blot in total extracts of sea urchin eggs, cultured in the presence or absence of the drug. In unfertilized eggs (Fig. 2A), phosphorylated-MAPK/ERK level was readily detectable, indicative of the high level of the MAPK/ERK pathway activity in these cell cycle-arrested cells (see introduction). Addition of U0126 at 20 or 60 μM in the incubation medium induced a rapid and complete disappearance of the phosphorylated, and therefore active, form of MAPK/ERK (Fig. 2A, upper panels, P-MAPK/ERK). Using an antibody directed against the whole protein, we verified that the level of MAPK/ERK protein remained constant after U0126 treatment (Fig. 2A, lower panels, MAPK/ERK).

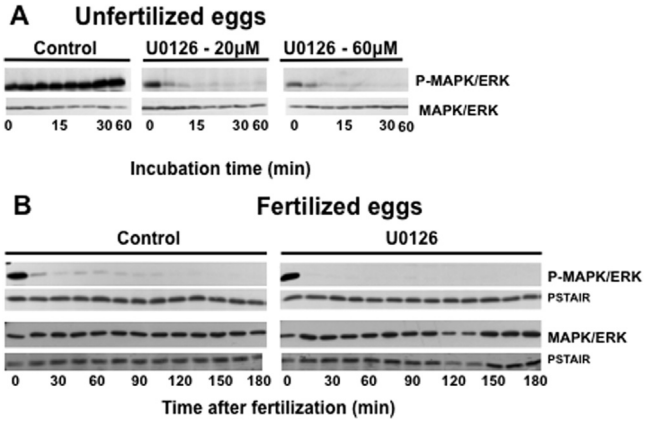

Fig. 2. Effect of U0126 on MAPK/ERK phosphorylation. (A) Batches of unfertilized eggs were incubated in the absence (control) or presence of U0126 (20 μM or 60 μM) (U0126-20; U0126-60). Total extracts of embryos were prepared every 5 min until 30 min and at 60 min as indicated in Material and Methods. Proteins were resolved by 12% SDS-PAGE and subjected to western blotting analysis using phospho-MAPK antibodies (top panels) or MAPK/ERK antibodies(lower panels). (B) Fertilized embryos were incubated immediately (< 5 min) after fertilization in the absence (control) or presence of U0126 (60 μM) (U0126). Total embryo extracts were prepared and immuno-revealed using P-MAPK/ERK, MAPK/ERK and PSTAIR antibodies.

The phosphorylated MAPK/ERK level was then investigated in embryos after fertilization. The high level detected in unfertilized eggs abruptly decreased during the first 15 min following fertilization, as already reported by Ciapa's and Foltz's groups (see introduction). Thereafter the phosphorylated, and therefore active, MAPK/ERK level remained very low. Among 7 experiments performed on different sets of parents, the remaining signal was hardly detectable ranging from 0.1% to 10% of the signal measured in unfertilized eggs, thus involving a small fraction of the enzyme. Interestingly, the low level of phosphorylated MAPK/ERK usually showed an oscillatory pattern, with a maximum of phosphorylation between 45 and 75 min (Fig. 2B; left panel). The addition of U0126 immediately after fertilization induced the complete disappearance of the phosphorylated MAPK/ERK signal (Fig. 2B, right panel). The presence of this fraction of MAPK/ERK activity is correlated with the time of action of U0126 on cell division, as defined above.

Altogether, these results demonstrate that the effect of the MEK inhibitor, U0126, was directly related to the inhibition of MAPK/ERK activity in sea urchin eggs. They show that a low level of MAPK/ERK phosphorylation was apparent after fertilization, exhibiting an oscillatory pattern, consistent with the window of action of U0126 on cell cycle division.

3.3. MAPK/ERK inhibition on the evolution of chromatin and microtubule apparatus

The effect of MAPK/ERK inhibition by U0126 on cell cycle progress was analysed at a cellular level. The drug did not affect fertilization *per se* since elevation of the fertilization membrane normally occurred (data not shown). The evolution of chromatin morphology was followed after Hoechst dye staining by observation using fluorescent microscopy (Fig. 3A). No differences were observed between control and drug treated embryos during the first 60 min post-fertilization: the movement of the female and male pronuclei, their fusion, male pronucleus decondensation, the formation of the zygotic nucleus and the initial step of chromatin condensation all appeared morphologically normal and in time in both conditions. After 60 min however, the treated embryos showed significant abnormality in their chromatin evolution (Fig. 3A, lower panels). Although the chromatin seemed properly condensed, the condensed chromosomes were found unequally dispersed at the centre of the embryo corresponding to the site of the former nucleus. They were never found aligned onto a metaphase plate or symmetrically migrating towards the embryo poles.

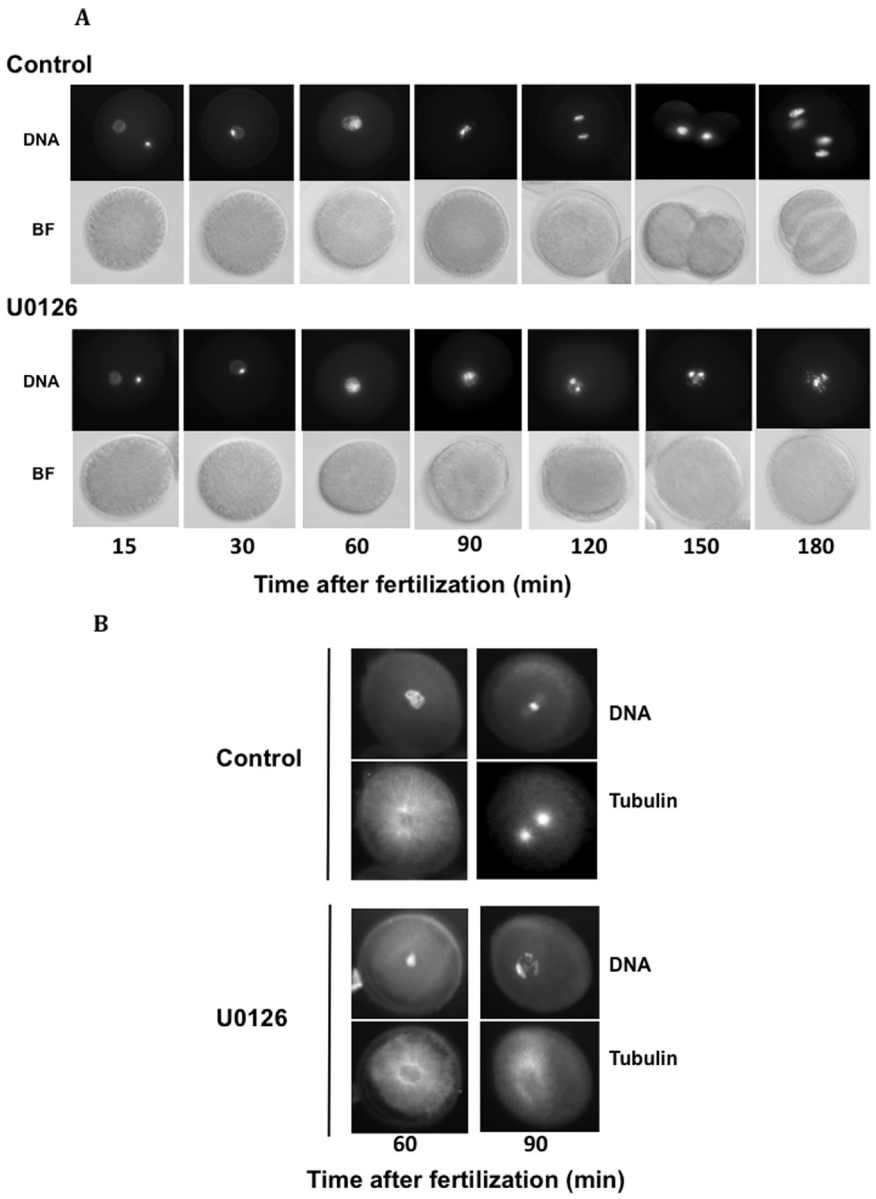

Fig. 3. Effect of U0126 on chromatin and microtubules morphology evolution. (A) Chromatin morphology (DNA) and cell division (BF) in control (upper panels) and 60 μM U0126-treated embryos (lower panels) are observed after fixation and Hoechst labelling of batches of embryos taken at the indicated times post-fertilization, by bright field and fluorescent microscopy. (B) Control and 60 μM U0126-treated embryos were taken at 30, 60, 90 and 120 min after fertilization for fixation and labelling, as described in material and methods. Chromatin and microtubules were labelled with Hoechst (DNA) and Alexa Fluor-conjugated alpha-tubulin antibody (Tubulin).

Fig. 3B shows the evolution of the spindle formation in control and U0126-treated embryos after immuno-staining with an alpha-tubulin antibody. While control embryos showed the presence of two microtubule polymerisation sites (corresponding to the location of the duplicated centrosomes) at each pole of the nucleus at 60 min, U0126-treated embryos displayed several aster formations all around the nucleus. Control embryos subsequently contained a well-organized spindle between the two centrosomal asters on which the chromatin aligned (at 90 min) and then partitioned. U0126 embryos never displayed spindle formation but rather microtubule formation, around the different pools of condensed chromatin (Fig. 3B, lower panel).

The role of MAPK/ERK activation during the first hour after fertilization was therefore related to the proper chromatin repartition, and formation of a normal microtubular network during mitosis.

3.4. MAPK/ERK inhibition on DNA replication

The necessary period of MAPK/ERK activity appeared to take place during the first hour after fertilization (see Fig. 1B), at the time of S-phase. The effect of MAPK/ERK inhibition was then tested on DNA replication by measuring the *in vivo* radiolabelled thymidine incorporation into DNA, in control and U0126-treated embryos, at different times after fertilization (Fig. 4). In control embryos, as expected, incorporation was detected at 30 min post-fertilization that increased up to 60 min. After plateauing until 90 min, the incorporation doubled again at 120 min post-fertilization, in concordance with entry into the second embryonic division. As a control, incorporation of thymidine into DNA was totally inhibited by aphidicolin, a DNA polymerase I inhibitor, indicating that the thymidine incorporation measurement reflected the DNA synthesis activity of the embryos. When the embryos were treated with 60 μM U0126, thymidine incorporation into DNA was unaffected until 90 min post-fertilization and remained aphidicolin sensitive. Therefore, the existence of the fraction of activated MAPK/ERK after fertilization did not interfere with normal DNA replication.

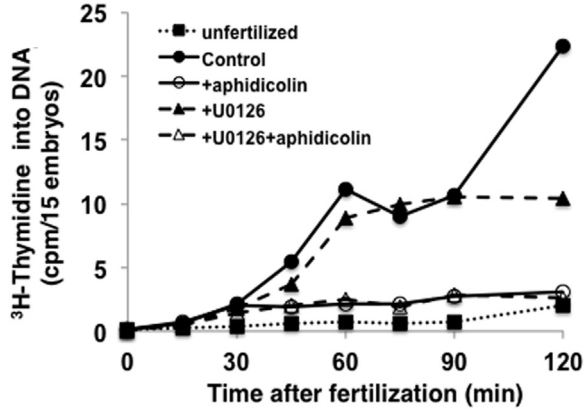

Fig. 4. Effect of U0126 on DNA replication. The rate of in vivo DNA replication was monitored by the kinetics of [methyl^{3}H] thymidine incorporation DNA. *S. granularis* eggs (5% cells/vol solution in FSW) were labelled for 10 min in the presence of [methyl-^{3}H] thymidine (10 µCi/ml). Immediately after fertilization, embryos were incubated in the absence (circles) or presence (triangles) of 60 µM U0126. At different times, cytosoluble fractions were prepared from 20 µl pelleted-embryos and radioactivity incorporation into TCA-precipitated material was determined. The curve was obtained from embryos issued from a single set of parents and was representative of 2 independent experiments.

3.5. MAPK/ERK inhibition on cyclin B production

The essential protein that is required to enter M-phase after fertilization is cyclin B (Hunt, 2002). Cyclin B levels were quantified by western blotting in control and U0126-treated embryos, at different times post-fertilization (Fig. 5). As already well documented, the level of cyclin B was low in unfertilized eggs. After fertilization, in control embryos, the level of cyclin B progressively increased until 60 min post-fertilization and then rapidly augmented, reaching its highest level at around 90 min. Cyclin B level then abruptly decreased, due to the rapid degradation of the protein, required to allow exit from metaphase. A second peak of cyclin B accumulation was observed at 150 min revealing the occurrence of the second division.

In U0126-treated embryos, no difference in the levels or kinetics of cyclin B was observed until 60 min after fertilization. After 60 min, cyclin B accumulation in treated embryos was significantly slowed down and remained 40–60% beneath the control level. Furthermore, no significant decrease could be detected during the following 2 h, indicating that the degradation of cyclinB did not occur in treated eggs, in concordance with the absence of division.

Altogether, inhibition of MAPK/ERK activity impaired the oscillations of cyclin B level, suggesting that MAPK/ERK activity was necessary for full synthesis of cyclin B and for cyclin B degradation.

3.6. MAPK/ERK inhibition on CDK1/Cyclin B activation

Appropriate dynamics of cyclin B production after fertilization is required for the activation of CDK1/cyclin B complex, the universal M-phase promoting factor (reviewed in Murray, 2004). The kinetic of CDK1/cyclin B activation was therefore analysed in U0126-treated embryos following fertilization. *In vivo,* CDK1/cyclin B activity can be monitored by quantifying the endogenous phosphorylation level of one of its specific substrates, PP1Cα (Lewis et al., 2013). The PP1Cα phosphorylation status was analysed by western blotting of total extracts from embryos, taken at different times after fertilization (Fig. 6). In control embryos, a peak of PP1Cα phosphorylation was

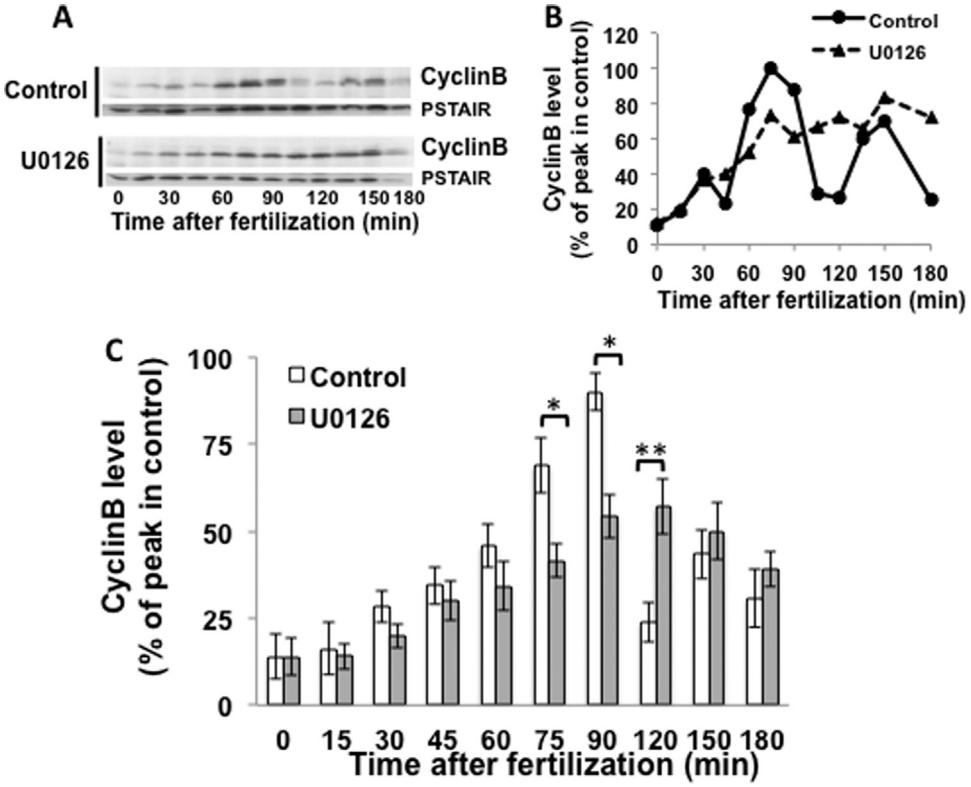

Fig. 5. Effect of U0126 MAPK/ERK on cyclin B level. (A) Total amount of cyclin B in extracts from control or U0126-treated embryos of *S. granularis*, obtained at indicated times post-fertilization, was monitored by western blotting using anti-cyclin B (top panels). CDK1 immuno-labelling with PSTAIR antibody was used as a loading control for western blot (bottom panels). (B) Quantification of the results obtained from control (circles) or U0126-treated (triangles) embryos. Cyclin B level, the sum of the two immuno-labelled bands, was normalized against PSTAIR level and expressed as a percentage of the maximal value reached at M-Phase in control embryos. (C) Quantitation of cyclin B level obtained from untreated (white boxes) and U0126-treated (grey boxes) embryos at the indicated time following fertilization. Cyclin B level was normalized against PSTAIR level and expressed as a percentage of the maximal value reached at M-Phase in control embryos. Vertical bars represent Standard Error of the Mean values (+/- SEM) obtained in 6 independent experiments. *Indicates a significant difference in cyclin B level in untreated embryos in comparison to the U0126-treated one (Student's test, $p < 0.05$).

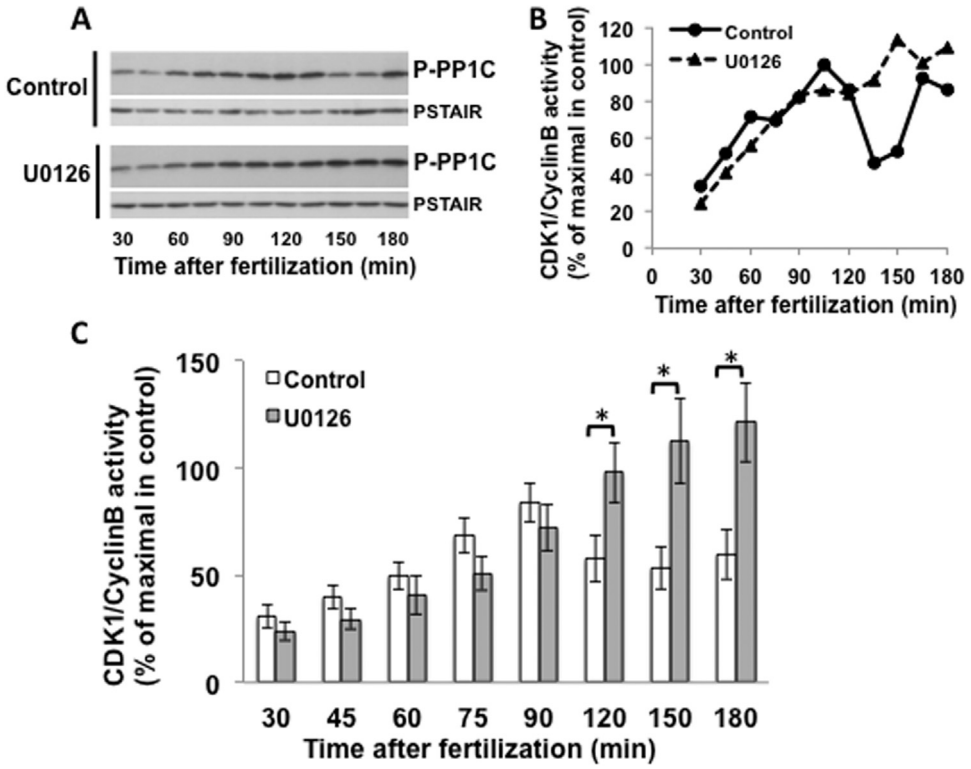

Fig. 6. Effect of U0126 on CDK1/Cyclin B activation. (A) Fertilized embryos were incubated immediately (< 5 min) after fertilization in the absence (control) or presence of U0126 (60 μM) (U0126). Aliquots of total extracts from 20 μl pelleted embryos taken at indicated times post-fertilization were resolved by 12% SDS-PAGE and subjected to western blotting analysis using phospho^{320}T- PP1Cα (top panels). CDK1 immuno-labelling with PSTAIR antibody was used as a loading control for western blot (bottom panels). (B) Quantification of the results obtained from control (circles) or U0126 treated (triangles) embryos. Phospho^{320}T-PP1Cα levels were normalized against PSTAIR levels obtained at the same time and expressed as a ratio of the value obtained with unfertilized eggs. (C) Quantitation of Phospho^{320}T-PP1Cα levels obtained from untreated (white boxes) and U0126-treated (grey boxes) embryos at the indicated time following fertilization. Phospho320T-PP1Cα level was normalized against PSTAIR level and expressed as a percentage of the maximal value reached at M-Phase in control embryos. Vertical bars represent Standard Error of the Mean values (+/- SEM) obtained in 7 independent experiments. *Indicates a significant difference in Phospho^{320}T-PP1Cα level in untreated embryos in comparison to the U0126-treated one (Student's test, $p < 0.05$).

observed at 90 min post-fertilization, reflecting the time of the first M-phase. When embryos were treated with U0126, PP1Cα phosphorylation increased until 90 min post-fertilization, with a comparable range and kinetic to those observed in control embryos. This result reveals a normal activation of CDK1/cyclin B.

On the other hand, after 90 min, the level of PP1Cα phosphorylation differed significantly between control and treated embryos. In control embryos, PP1Cα phosphorylation rapidly decreased, reflecting the normal inactivation of CDK1/cyclin B required for metaphase exit and cytokinesis. In U0126-treated embryos, PP1Cα phosphorylation remained high, indicating that CDK1/cyclin B remained fully active.

Altogether, the inhibition of MAPK/ERK activity did not affect normal CDK1/cyclin B activation, but it impaired its inactivation at the end of mitosis. This is in accordance with the absence of cyclin B degradation (see Fig. 5) and the arrest in cell cycle induced by U0126 treatment.

3.7. MAPK/ERK inhibition on 4E-BP level

The accumulation of cyclin B in sea urchin embryos after fertilization depends in part on the mTOR-dependent degradation of 4E-BP, the cap-dependent translation inhibitor (see introduction). The level of 4E-BP was therefore analysed by western blotting, in U0126-treated embryos at different times after fertilization (Fig. 7). In control embryos, as already reported (Salaun et al., 2003), fertilization induced the rapid and almost complete disappearance of 4E-BP during the first 15 min. The protein then remained at a basal level for at least 180 min. In U0126-treated embryos, the 4E-BP drop after fertilization is similar to the control, reaching the same low basal level in 15 min. However, as early as 60 min after fertilization the protein re-appeared in the treated embryos, reaching a steady state level at around 90 min.

This result indicates that MAPK/ERK activation at the time of S-phase is required to maintain a low 4E-BP level during the mitotic cell division.

3.8. MAPK/ERK inhibition on global protein synthesis

We then analysed the effect of MAPK/ERK on global protein synthesis activity. [^{35}S] L-methionine incorporation into proteins after 10 min pulse labelling was analysed in embryos during the first 2 h following fertilization, in the presence or absence of U0126 (Fig. 8). In control embryos, protein synthesis activity progressively increased from 15 min to 60 min after fertilization, as already reported (Belle et al., 2011). At that time, the activity reached a plateau and was 30 times higher than in unfertilized eggs. The presence of U0126 impaired the increase in protein synthesis activity, as early as 15 min after fertilization. At the time when the control embryos reached the plateau of activity, the increase in drug-treated embryos never exceeded 10 times the protein synthesis activity of unfertilized eggs. It must be focused that U0126 effect on protein synthesis was observed as early as 15 min after fertilization, at a time when the drug has not yet affected the 4E-BP level (compare Figs. 7 and 8).

Therefore, inhibition of the MAPK/ERK activity after fertilization triggered a significant decrease in the fertilization-induced stimulation of protein synthesis.

4. Conclusion

While it is well established that the MAPK pathway plays a major role in regulating cell proliferation at the level of the restriction point

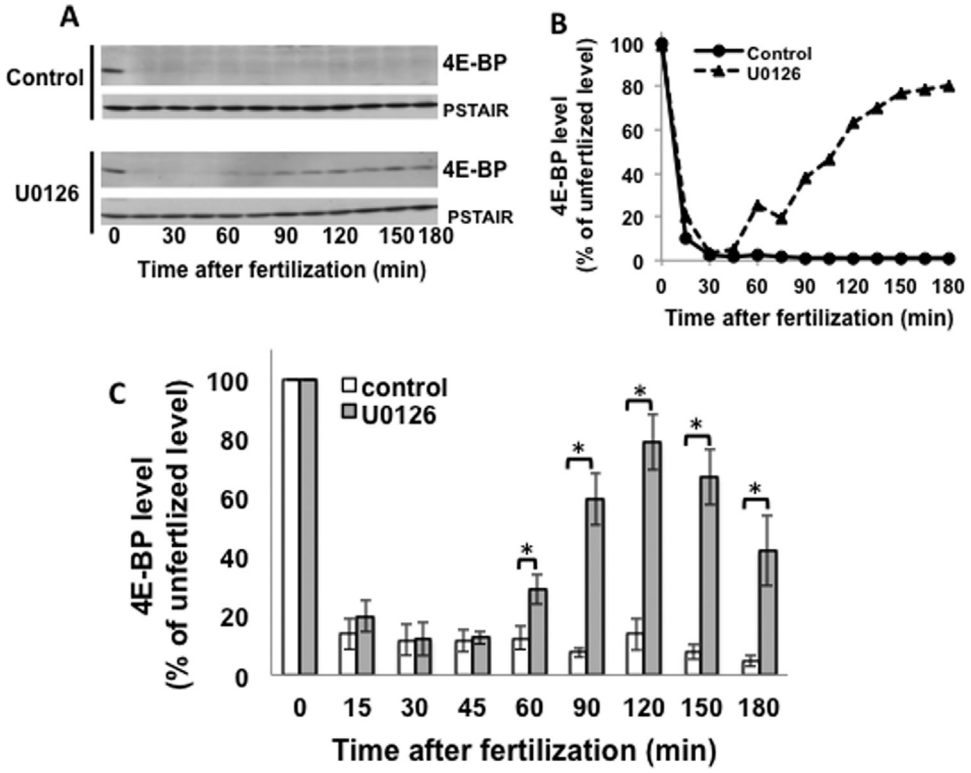

Fig. 7. Effect of U0126 on 4E-BP level. (A) Total amount of 4E-BP in extracts from control or U0126-treated embryos of *S. granularis*, obtained as in Fig. 3, was analysed by western blotting using anti-sea urchin 4E-BP antibodies (top panels). CDK1 immuno-labelling with PSTAIR antibody was used as a loading control for western blot (low panels). (B) Quantification of the results obtained in (A) from control (circles) or U0126 treated (triangles) embryos showed. 4E-BP amount was normalized against PSTAIR level and expressed as a percentage of the value obtained with unfertilized eggs. (C) Quantitation of 4E-BP abundance obtained from untreated (white boxes) and U0126-treated (grey boxes) embryos at the indicated time following fertilization. 4E-BP amount was normalized against PSTAIR level and expressed as a percentage of the value obtained with unfertilized eggs. Vertical bars represent Standard Error of the Mean values (+/- SEM) obtained in 6 independent experiments. *Indicates a significant difference in 4E-BP amount in untreated eggs in comparison to the U0126-treated one (Student's test, $p < 0.05$).

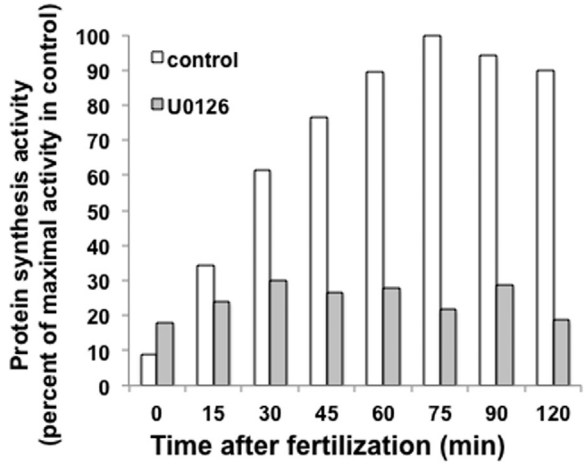

Fig. 8. Effect of U0126 on protein synthesis activity. The rate of in vivo protein synthesis was monitored by the kinetics of [^{35}S]methionine incorporation into proteins. *S. granularis* eggs (5% cells/vol solution in FSW) were metabolically labelled in the presence of [^{35}S] L-methionine. Immediately after fertilization, embryos were incubated in the absence (circles) or presence (triangles) of 60 μM U0126. At different times, cytosoluble fractions were prepared from 20μl-pelleted embryos and radioactivity incorporation into TCA-precipitated proteins was determined. The curve was obtained from embryos issued from a single set of parents and was representative of 2 independent experiments.

(G0/G1 transition), its involvement in the control of mitosis is still an area of conjecture. Taking advantage of the powerful model of sea urchin *S.granularis* embryos to study the regulatory mechanisms that govern the cell cycle, we report here that MAPK/ERK activity is actually required to ensure proper cell mitotic division after the G1-phase.

Our results show that after the precocious, fertilization-induced, huge drop in MAPK/ERK activity a limited fraction of MAPK/ERK, less than 10% of the signal measured in unfertilized eggs, is activated through its phosphorylation by MEK during the first hour following fertilization. These results were obtained using the accepted and widely used pharmacological MEK inhibitor U0126. The specificity of U0126 in our conditions was further ascertained by experiments performed using PD98059, another specific MEK1 inhibitor. Comparable results on cell cycle as well as on MAPK/ERK phosphorylation were obtained (data not shown), thus strongly arguing for the requirement of MAPK/ERK pathway during cell division in sea urchin.

These data confirm studies in *Paracentrotus lividus* by Ciapa and coll. (Zhang et al., 2005), and rules out a previous report by Kumano and coll. (Kumano et al., 2001). We assume that the discrepancy could be due to the small MAPK/ERK fraction concerned.

We further demonstrate the requirement of this activated MAPK/ERK fraction for mitotic division. Precise determination of the time window of when the activation is required indicates that MAPK/ERK activation should occur around the first hour after fertilization, that is for *S.granularis* embryos about 30 min before the CDK1 peak of activity occurring around 90 min post-fertilization and 60 min before the first embryonic division. This result accurately specifies the timing for a required MAPK/ERK activity during the first mitotic division.

Interestingly, our results further suggest that MAPK/ERK activation is not specific to the fertilization signal, but is required at each mitotic division, since the incubation with MEK inhibitor at the entry of the second cell cycle after fertilization, *i.e.* around 90–120 min after fertilization, had no effect on the first division but impaired the second embryonic division.

Since the early embryonic divisions are free from transcriptional

regulation, MAPK/ERK transcriptional substrates implicated in G1-phase (see introduction) are not involved in the MAPK/ERK control on embryonic cell division. The role of MAPK/ERK must then be investigated at translational or post-translational levels.

Protein synthesis is required at each cell cycle for cyclin B synthesis, its association to CDK1 and for the activation of the CDK1/cyclin B complex (Epel, 1990). There is a large amount of evidence showing that fertilization leads to activation of the mTOR signalling pathway that in turn induces phosphorylation of 4E-BP, and its subsequent degradation. This releases eF4E from its repressor 4E-BP, allows its association with eIF4G and consequently mRNA translation (Cormier et al., 2003, 2016; Chasse et al., 2016). Our present data demonstrate a major role for the MEK/MAPK/ERK cascade in global protein synthesis following fertilization. It can therefore be suggested that the fertilization-induced increase in global protein synthesis results from the cooperative actions of both the mTOR and MAPK/ERK pathways. Since the inhibitor of MAPK/ERK activity did not affect the level of 4E-BP immediately after fertilization, the target of MAPK/ERK to regulate global protein synthesis remains to be elucidated independently of mTOR signalling.

Paradoxically, the inhibitor did not affect initial cyclin B synthesis during the first hour following fertilization nor the time and the intensity of CDK1 activation. The fact that cyclin B accumulation escaped MAPK/ERK regulation of global protein synthesis may be related to the two recently described independent mechanisms that control cyclin B mRNA translation (Chassé et al. 2016). After the first hour the inhibitor impaired the complete synthesis of cyclin B without affecting the full CDK1/Cyclin B activation, thus demonstrating that a limited level of cyclin B is sufficient to obtain full CDK1/cyclin B activation. Finally, MAPK/ERK inhibition prevented the degradation of cyclin B and the decrease of CDK1 activity, therefore targeting a cell cycle checkpoint. Importantly, the inhibitor did not impair DNA replication suggesting a role for MAPK/ERK independent of the cell cycle replication checkpoint (Weinert et al., 1994). On the other hand, the inhibitor affects the spindle formation. Therefore it probably activates the spindle checkpoint (Malumbres, 2014) resulting in cell cycle arrest.

Taken together, our data clearly show that the mechanism regulating cyclin B accumulation depends on two different regulatory pathways. The first one, activated immediately after fertilization, is insensitive to MAPK/ERK inhibition and must essentially rely on the mTOR-signalling pathway (Chassé et al., 2016). The second one, observable at 60–90 min post-fertilization, depends on MAPK/ERK activity and leads to a peak of cyclin B level.

An interesting working hypothesis can be proposed for the physiological role of this full MAPK/ERK-dependent cyclin B synthesis. Our morphological studies precisely reveal that inhibition of the MAPK/ERK pathway impairs chromatin distribution and microtubule network organisation as soon as 60 min following fertilization. This timeline fits well with the action window of the inhibitor on cell division. Accordingly, it was reported that sea urchin embryos showed profound mitotic spindle defects when cultured in the presence of MAPK/ERK inhibitors during the first division (Kumano et al., 2001; Pesando et al., 1999; Zhang et al., 2005). Altogether these data suggest that the initial target for MAPK/ERK could be in the vicinity of the early microtubular structure formation (centrosomes, centromeres, kinetochores). Interestingly, the kinase MNK, a target for MAPK/ERK and responsible for the phosphorylation of the translation initiation factor eIF4E, was also shown to localize to mitotic structures in proliferating HeLa cells (Rannou et al., 2012). Furthermore, in *Xenopus* embryos, it was reported that the bipolar spindle formation depends on the proper perinuclear localization of cyclin B (Yoshitome et al., 2012). Finally, it has been observed that a pool of cyclin B accumulates in the nucleus of sea urchin embryos early after fertilization, mainly associated with chromatin (Geneviere-Garrigues et al., 1995). It can therefore be proposed that the final target for the fraction of activated MAPK/ERK should be a specific cyclin fraction, for which the localization/synthesis would be necessary for correct spindle/chromatin interaction.

How could embryos treated with MAPK/ERK inhibitor retain relatively high CDK1/cyclin B activity and a sustained elevated cyclin B level? These features are characteristic of the spindle checkpoint activation, which prevents mitotic cells from exiting mitosis in the presence of unattached or improperly attached chromosomes by blocking the activity of the anaphase-promoting complex (APC). APC is responsible for cyclin B degradation and thus CDK1/cyclin B inactivation, allowing the metaphase-anaphase transition (reviewed in Musacchio (2015)). Thus, we propose that the target for MAPK/ERK pathway could be (or be related to) an early sensor for a specific path of the spindle checkpoint acting at the time of chromatin first attachment to the microtubular network. Interestingly, at the time when morphological anomalies became observable in treated embryos, the global level of 4E-BP, which is low following fertilization, gradually increases and the protein synthesis activity is correlatively maintained at a very low level. This suggests that this represents an additional MAPK/ERK-dependent response triggered with checkpoint activation, a survival response to save energy for the cell by keeping a reduced protein synthesis activity.

In conclusion, we propose a mechanism for the role of MAPK/ERK at the beginning of sea urchin early cell divisions (Fig. 9). MAPK/ERK activity is required to stimulate the global protein synthesis activity by a yet unidentified mechanism, which acts in collaboration with the well-demonstrated mTOR-signalling pathway. The MAPK/ERK dependent activation of protein synthesis would induce, either directly or via the synthesis of a yet unidentified factor (X), the accumulation of a specific fraction of cyclin B protein. This specific fraction of cyclin B would be responsible for the proper attachment of chromatin to the microtubule network at the end of the S-phase. In case of a MAPK/ERK activation default, a checkpoint would be triggered to block cell cycle progression and would involve the reappearance of the translational inhibitor 4E-BP, required for energy saving by maintaining low protein synthesis activity.

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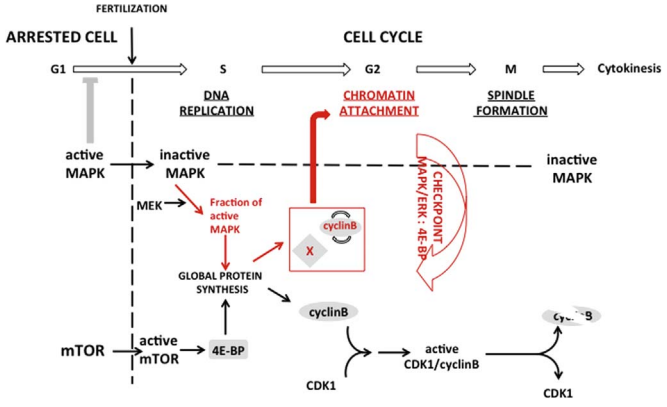

Fig. 9. Hypothetical MAPK/ERK signalling pathway during mitotic division in sea urchin embryo. The global MAPK/ERK activity is inactivated after fertilization to trigger resumption of the cell cycle of the G1-arrested eggs. A fraction of activated MAPK/ERK participates together with the mTOR dependent 4E-BP degradation to the activation of global protein synthesis. This MAPK/ERK activated fraction specifically triggers the synthesis of a protein (X), which allows the accumulation/localization of a fraction of cyclin B implicated in chromatin attachment to microtubular structures. This yet unidentified MAPK/ERK target is part of a checkpoint, acting upon 4E-BP turnover to allow the control of protein synthesis in case of abnormal chromatin/microtubule attachment.

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