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Review

Cell cycle arrest and activation of development in marine invertebrate deuterostomes

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ABSTRACT

Like most metazoans, eggs of echinoderms and tunicates (marine deuterostomes, there is no data for the cephalochordates) arrest awaiting fertilization due to the activity of the Mos/MEK/MAPK cascade and are released from this cell cycle arrest by sperm-triggered Ca2+ signals. Invertebrate deuterostome eggs display mainly three distinct types of cell cycle arrest before fertilization mediated by potentially different cytostatic factors (CSF): one CSF causes arrest during meiotic metaphase I (MI-CSF in tunicates and some starfishes), another CSF likely causes arrest during meiotic metaphase II (amphioxus), and yet another form of CSF causes arrest to occur after meiotic exit during G1 of the first mitotic cycle (G1-CSF). In tunicates and echinoderms these different CSF activities have been shown to rely on the Mos//MAPK pathway for establishment and on Ca2+ signals for their inactivation. Despite these molecular similarities, release of MI-CSF arrest is caused by APC/C activation (to destroy cyclin B) whereas release from G1-CSF is caused by stimulating S phase and the synthesis of cyclins. Further research is needed to understand how both the Mos//MAPK cascade and Ca2+ achieve these tasks in different marine invertebrate deuterostomes.

Another conserved feature of eggs is that protein synthesis of specific mRNAs is necessary to proceed through oocyte maturation and to maintain CSF-induced cell cycle arrest. Then activation of development at fertilization is accompanied by an increase in the rate of protein synthesis but the mechanisms involved are still largely unknown in most of the marine deuterostomes. How the sperm-triggered Ca2+ signals cause an increase in protein synthesis has been studied mainly in sea urchin eggs.

Here we review these conserved features of eggs (arrest, activation and protein synthesis) focusing on the non-vertebrate deuterostomes.

Contents

1. Introduction

The mature oocyte (or egg) contains all the factors necessary to support early development but embryonic development is halted until fertilization. It has been known for more than 30 years that transient Ca2+ increases in the egg are the ubiquitous signals used

Abbreviations: MAPK, mitogen-activated protein kinase; MPF, maturation promoting factor; APC/C, anaphase promoting complex; CSF, cytostatic factor; eIF2 – eukaryotic initiation factor 2, 4E-BP – eIF4E (eukaryotic initiation factor 4E) binding protein.

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to stimulate development in metazoans [1]. Such cell cycle arrest of the egg is mediated by an activity discovered in *Rana pipens* amphibian oocytes called cytostatic factor [2]. Despite the universality of CSF and its inactivation by Ca2+, CSF arrest occurs at different time points during maternal meiosis: metaphase II in vertebrates and cephalochordates [21], metaphase I in many protostomes and tunicates, and interphase (mostly G1) in cnidarian, echinoderms and some protostomes (mollusks).

Because of these differences in cell cycle arrest at the end of oocyte maturation there are inconsistencies in the terminology surrounding oocytes/eggs/fertilized eggs (and zygotes). For example, some authors, including the entire mammalian field, use the criteria that exit from the meiotic cell cycle (when the pronucleus forms) defines the transition from oocyte to egg. Thus the metaphase II oocyte awaits fertilization in the mammalian field and the fertilized oocyte becomes a zygote following pronuclear fusion. However, other authors prefer to use "egg" to describe the female gamete that can be fertilized regardless of the state of meiotic maturation. Thus, in the mammalian field, the fertilized egg becomes a zygote following pronuclear fusion (the karyogamy). Here we will use the egg nomenclature to refer to the mature female gamete at the moment of fertilization. Likewise there are some differences in the usage of zygote. Like Wessell we prefer to use zygote to define the moment of union between the male and female pronuclei rather than fusion between the sperm and the egg [3].

Eggs and embryos of many marine invertebrate deuterostome species have very favorable optical properties and are easy to manipulate in the laboratory (i.e., external fertilization and development). For these reasons eggs of marine invertebrate deuterostomes have been used in embryological studies for more than a century. While first documented embryonic development was of sea urchin embryos [4], the first experimental approaches in embryology were performed using ascidian embryos [5] (reviewed by Fischer [6]). The precise details of ascidian embryogenesis were documented in depth later by Conklin [7]. Furthermore, thanks to the copious amount of gametes recovered, these eggs have been useful material for biochemical studies since the late 40's (sea urchin [8], starfish [9]) and several processes of oocyte maturation, CSF arrest and meiotic cell cycle were first characterized using starfish oocytes ([10], reviewed in [11]) before being confirmed in vertebrates. What was once useful for biochemistry is now useful for proteomic studies for similar reasons. Today, there are genomic and transcriptomic resources for several species of tunicates (ascidians: www.anisse d.cnrs.fr, appendicularian:http://www.genoscope.cns.fr/externe/GenomeBrowser/Oikopleura/) or echinoderms (sea urchin: www.spbase.org/SpBase/, starfish: *Patiria miniata*, www.echinobase.org/Echinobase/PmBase) which have opened the way to specifically modify each gene via morpholino oligonucleotide injections or TALEN-mediated deletions/mutations [12]. Finally, the success of transgenesis in these species permits the visualization of the localization and/or activity of GFP-tagged proteins and to use fluorescent molecular imaging techniques in the living egg or embryo.

Oocyte development is devoted to exchange genetic material between homolog chromosomes and decrease chromosomal number during meiosis but more importantly to producing and storing all constituent necessary to support early development until zygotic transcription can take over. During vitellogenesis, active transcription and massive protein synthesis generate stockpiles of maternal mRNAs and proteins. Then transcription ceases at the resumption of oocyte maturation and tightly regulated translation of these maternal mRNAs drives the oocyte through meiosis until CSF arrest occurs. The egg thus hosts the maternal proteome and transcriptome that will drive cleavage of the early embryo. For example, artificially activating enucleated sea urchin eggs (thus, activation without sperm or gynogenesis) can remarkably result in cell division and formation of a blastula stage embryo [13]. Furthermore, inhibiting transcription in *Xenopus* embryos does not affect development up to the blastula stage [14]. These experiments clearly reveal the maternal nature of early embryonic development. Because of the ease of maturing starfish oocytes *in vitro*, the starfish model has contributed enormously to the understanding of such processes in metazoans [15–17]. Unfortunately no other marine invertebrate deuterostome oocytes are so easily amenable to *in vitro maturation*, however despite this limitation there are some articles detailing oocyte maturation in ascidians [18,20], and sea urchins [19].

Oocyte maturation and the onset of development are two developmental windows during which regulated mRNA translation is of utmost importance. Indeed protein translation can both drive progression through meiotic (and early mitotic) divisions and arrest cell cycle progression in the mature egg. Even though the regulation of CSF by the Mos//MAPK(ERK1/2) pathway is universal [23], large divergences have emerged downstream of ERK1/2 to result in the different outcomes of CSF observed in echinoderm or tunicate eggs. Further diversity in CSF arrest points is found among starfishes suggesting an important selective pressure on the different starfish species which adapted by deploying different life cycles.

In this review, we will first describe the different types of CSF arrest observed in marine invertebrate deuterostome eggs. Then we will briefly describe how fertilization Ca2+ signals start and stop. We will then emphasize how Ca2+ inactivates CSF to trigger a mitotic cell cycle oscillator repressed in the egg. Finally we will discuss some aspects of the regulation of mRNA translation in the egg. Biological models such as the mouse, *Xenopus* or *Drosophila* are well documented in recent reviews [24–27]. Here we will address advances on egg activation mechanisms provided by studies on marine deuterostomes such as echinoderms and several chordates (mainly tunicates).

2. CSF arrest in marine deuterostomes is established and maintained by the Mos//MAPK pathway

Fig. 1 depicts all the CSF arrests observed in marine invertebrate deuterostomes. CSF arrest point is very conserved in sea urchin species which arrest at G1 (*Paracentrotus lividus* [28], *Sphaerechinus granularis* [29], *Lytechinus variegatus*, *L. pictus*, *Strongylocentrotus purpuratus* [30]) and in all tunicates, which arrest at metaphase I (*Ciona intestinalis* [48], *Ascidiella aspersa* [59], *Phallusia mammillata* [41], *Boltenia villosa* [20], *Phallusia nigra* [74], *Halocynthia roretzi*, *Styela plicata*). In contrast, CSF arrest points diverge much more within asteroids with some starfish species (*Asterina pectinifera* [31,32], *P. miniata* [33]) arresting at G1, while *Astropecten aranciacus* and *Marthasterias glacialis* [34,35] arrest at G2 (reviewed in [15]). The pacific purple starfish *Pisaster ochraceus* and the Japanese starfish *A. pectinifera* even display two cell cycle arrests : one at metaphase I and another at G1 [36–38]. Metaphase arrest is characterized by high MPF activity and the APC/C must be blocked to prevent destruction of cyclin, MPF inactivation and completion of meiosis. In contrast G1 arrested eggs are preloaded with all the initiation/replication complexes and are ready to proceed through S phase [28,32], but they are prevented from doing so because of the lack of cdc45 in the nucleus [31]. Beyond S phase progression, entry into mitosis is blocked by a separate mechanism that down regulates synthesis of cyclins [32].

Despite such divergence in the actions of CSF, CSF arrest relies on the Mos/MEK/MAPK(ERK1/2) pathway in almost all metazoan eggs studied (Fig. 1). Mos is a kinase signaling upstream of the MEK/MAPK pathway (Fig. 1) initially described as c-Mos, a proto-oncogene (moloney sarcoma). Its role as part of CSF was first revealed in *Xenopus* [40]. For an extended overview of CSF signaling in metazoans see [25–27,42,43]. Mos and its associated MAPK

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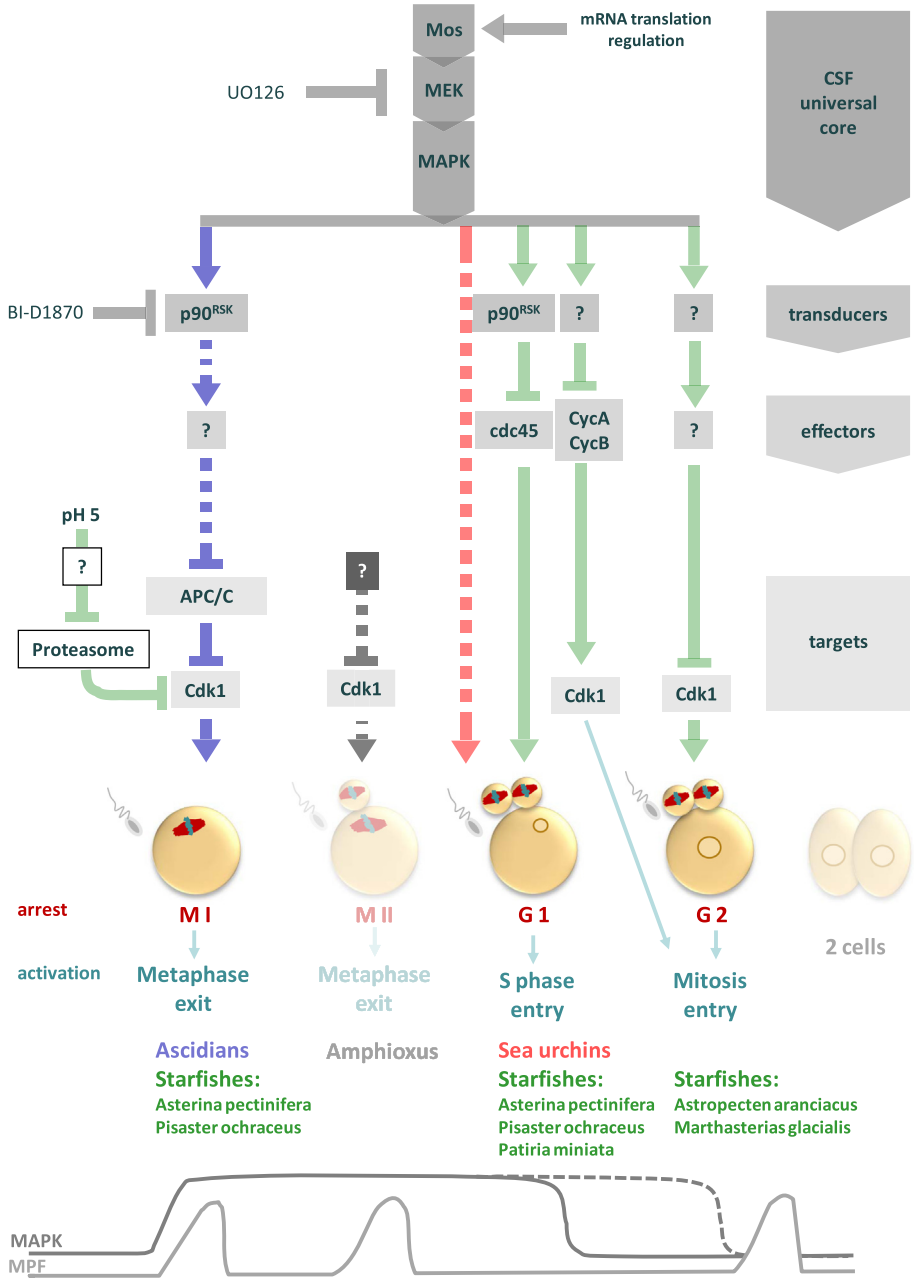

Fig. 1. Cytostatic signaling (CSS) in marine invertebrate deuterostomes. CSS signaling in ascidians is depicted in blue, sea urchins in red, starfishes in green and amphioxus in grey. The CSF universal core involves the Mos/MEK/MAPK signaling pathway. p90rsk has been shown to be a specific transducer of CSS in starfish. In ascidian, the p90rsk inhibitor BI-D1870 is able to activate unfertilized eggs suggesting that it might also be part of the CSS (McDougall et al., unpublished). Meta I arrest occurring in the gonad of starfishes is mediated by a low pH and the key step of Meta I arrest in these oocytes occurs after the polyubiquitination of cyclin B but before cyclin B proteolysis by the proteasome (see [36,37] for details). Potential effectors and targets of CSF arrest are depicted. The graph shows that MAPK activity is high during meiosis I and II whereas MPF peaks during M I, M II and mitosis.

activity (ERK1/2) are elevated during maternal meiosis I and II throughout the Eumetazoa: Cnidaria [44], Mollusc [45], Echiura [46], Echinoderms [47], Urochordates [48–50], Vertebrates [51,52]. Active MAPK is essential for completion of the meiotic cell cycle and repression of S phase [50,53,22]. It is also required for many of the diverse meiotic arrests in various organisms (for reviews see [54,55]), as well as for G1 arrest in eggs of starfish [47], sea urchin [56], jellyfish [57] and in meta-I arrest of sawfly [58] and tunicate eggs [50].

In every case, inactivation of MAPK by fertilization results in the release from CSF arrest and the subsequent entry into the embryonic cell cycles. In the absence of fertilization, down regulation of the Mos//MAPK pathway in the egg is sufficient to induce parthenogenetic development in the starfish (via Mos knock down, [22]) to relieve APC/C inhibition in ascidians (via MEK chemical inhibition, [50,59]) and to enter into first mitosis in sea urchins (MEK chemical inhibition [60]). Targets of MAPK have been characterized in the starfish egg where it was nicely shown that MAPK stimulates $p90^{rsk}$ which will in turn phosphorylate cdc45 to prevent its nuclear accumulation thereby preventing DNA replication [31]. Another arm of MAPK action is independent of $p90^{rsk}$ and inhibits the translation of maternal transcripts of cyclin A and B to prevent mitotic entry but the exact targets of MAPK are still unknown [32]. The targets of MAPK in ascidians are not yet known even though

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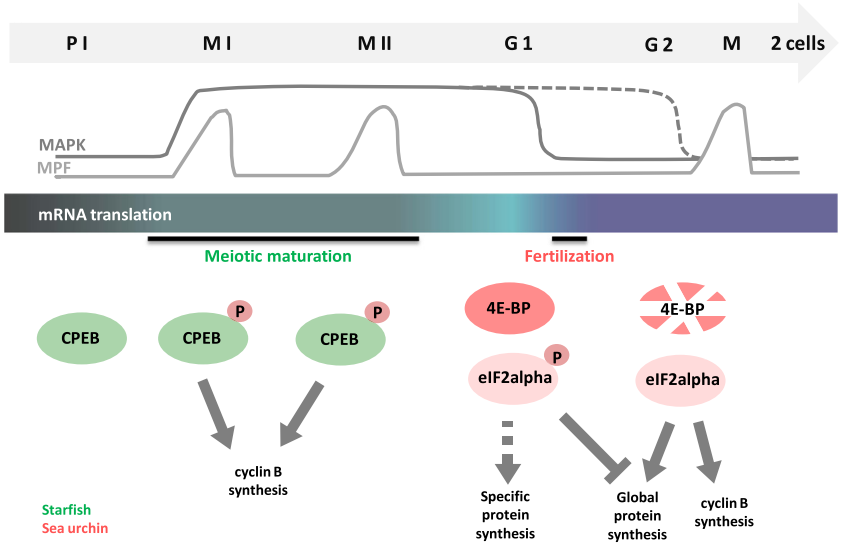

Fig. 2. Translational regulations during oocyte maturation and fertilization in echinoderms. Eggs of echinoderms can arrest at MI, G1 or G2 and MAPK remains high until fertilization in each species. Therefore in all echinoderms MAPK remains high until G1 except in G2-arrested species where MAPK remains elevated until G2. During oocyte maturation different types of protein synthesis are induced (colored horizontal bar), cyclin B is produced in starfish (green) under the control of CPEB phosphorylation which drives polyadenylation of cyclin B mRNAs. In G1-arrested sea urchin eggs (in red), the translational repressors 4E-BP and phospho-eIF2alpha are present to maintain specific protein synthesis of a potential G1-cytostatic factor. Fertilization in sea urchins provokes the permanent dephosphorylation of eIF2alpha and the degradation of 4E-BP, in order to allow global protein synthesis to increase.

MAPK inhibits the APC/C in these eggs [59]. However, it is interesting to note that the major target of the Mos//MAPK pathway in vertebrates that inhibits the APC/C in the egg (Erp1 in *Xenopus* and Emi2 in mammals) is not present in the ascidian genome. Although we know a great deal about Meta II arrest, this point of arrest is limited to the vertebrates (and cephalochordates [21]). Consistent with this finding, so far Erp1/Emi2 has only been identified in vertebrates. Thus, from both a cell cycle perspective and an "evolution of the cell biological mechanisms" perspective it would be interesting to identify the molecular nature of Meta I CSF, bearing in mind that Meta I arrest is one of the most prevalent forms of arrest point among the metazoan.

The fact that a repressed cell cycle oscillator is dormant in the unfertilized egg leads to another problem for eggs – to prevent parthenogenetic activation and teratoma formation. For example, when Mos is inactivated in the mouse this can lead to the formation of teratomas since the unfertilized oocytes do not arrest, and instead often divide and begin embryonic development in the mother [61], [62]. Mouse embryos form centrosomes spontaneously during early embryonic development and are thus prone to parthenogenesis [63]. In contrast, in marine deuterostomes the centrosome in the unfertilized egg is inactivated and cleavage depends entirely on paternal centrosome contribution thereby preventing parthenogenetic activation.

If the molecular pathways downstream of MAPK and the outcome of G1 and MI-CSF are very different, the inactivation of both CSF during fertilization relies on the sperm-triggered Ca2+ transients. This property of CSF brings a further constraint on CSF that is conserved not only in marine invertebrate deuterostomes but in all metazoans.

3. Starting and stopping the sperm-triggered Ca2+ oscillations

Back in 1901 Jacques Loeb got it surprisingly right. In an article entitled "Experiments on artificial parthenogenesis in annelids (Chaetopterus) and the nature of the process of fertilization" Loeb wrote on page 456 that "...the spermatozoon carries a catalytic substance into the egg, which accelerates the process that would otherwise start anyhow, but much more slowly" [64]. The process was egg activation and the catalytic substance became sperm factor which was finally identified as PLCzeta in mammals more than a hundred years later [65]. Loeb also hinted at the fact that unfertilized eggs would spontaneously activate then eventually die. This too turned out to be correct (unfertilized eggs eventually activate spontaneously and undergo apoptosis) but we do not have the space to cover that subject comprehensively (see [39,66,67]). Between Loeb's hypothetical sperm-borne "catalytic substance" in 1901 and identification of sperm factor in 2002, Ca2+ was discovered to be a universal activator of eggs. An increase in intracellular Ca2+ had been suspected to be responsible for egg activation since the discovery in 1974 by Steinhardt and Epel that challenging a sea urchin egg with a pulse of Ca2+ ionophore could induce parthenogenesis [68]. In a matter of a few years a sperm-triggered Ca2+ wave in the egg cytosol were first measured by Lionel Jaffe's group using medaka eggs [1]. These Ca2+ waves have now been recorded in mollusks, nemertean, sea urchins, tunicates and vertebrates eggs [69–71] and they are all thought to be induced by a sperm factor (or Loeb's "catalytic substance"). However the sperm factor(s) in tunicates and sea urchins are different from the mammalian PLCzeta and the identity of the sperm factor(s) in marine deuterostome eggs remains unknown [76,72].

The pattern of Ca2+ signals observed at fertilization varies depending on the cell cycle point of arrest of the mature egg [73]. A close look at the relationship between CSF arrest point and Ca2+ signals reveals that eggs which remain in meiosis for more than 20 min will elicit repetitive Ca2+ transients (mammals, tunicates, nemerteans) whereas eggs arrested in G1 (sea urchin, starfish, jellyfish) or that remain in M phase for less than 20 min after fertilization (like fish or frog) elicit a single Ca2+ transient [71]. Given that a sperm factor is delivered to egg cytosol for generating Ca2+ oscillations, this raises the question of how is the sperm factor inactivated in egg cytosol? Or in other words, how do the sperm-triggered calcium oscillations stop? It is intriguing to note that a clear correlation exists between the time that sperm triggered Ca2+ oscillations stop and the moment that the fertilized egg exits the meiotic cell cycle [49]. This correlation is not fortuitous. By maintaining the MPF activity elevated (through Δ90 cyclin B) the sperm-triggered Ca2+ oscillations continue indefi-

nitely in the ascidian Ascidiella *aspersa* [91]. This experiment has since been repeated in two other species of ascidian (Phallusia *nigra* [74], Phallusia *mammillata* [50]) as well as in mouse oocytes [75]. Conversely, inhibiting MPF in a MI-arrested ascidian egg prevents Ca2+ oscillations and only a single Ca2+ transient is elicited upon fertilization [76]. There is thus a strong link between high MPF in the egg and Ca2+ oscillations.

But what is the egg mechanism that controls the sperm factor? In the mouse it is now thought that the sperm factor PLCzeta [65] is sequestered into the pronucleus, thus causing the sperm-triggered Ca2+ oscillations to stop at meiotic exit [75]. This nicely explains how excess cyclin B prevented cessation of the Ca2+ oscillations, since it prevented pronucleus formation. However, pronucleus formation is not the likely mechanism causing the Ca2+ oscillations to stop in the fertilized ascidian egg. Enucleated ascidian eggs were injected with ascidian sperm cytosolic extract to directly test this hypothesis. In this experiment a pronucleus cannot form since there is no maternal or paternal DNA for it to form around but Ca2+ oscillations nevertheless stop at the correct time [76]. In the ascidian we favor a mechanism whereby the sperm-triggered Ca2+ oscillations are maintained by a cell cycle based mechanism that involves both sperm factor and the InsP3 receptor (ascidian eggs are not sensitive to cADPribose, McDougall personal observations). We suggest that sperm factor is kept active by Cdk1 and the Insp3 calcium release system is kept active by ERK1/2. This is based on our original observations on the temporal patterns of calcium oscillations, MPF, MAPK and InsP3 sensitivity [49].

4. Control of protein synthesis at fertilization: the potential actors

It has been long known that blocking protein synthesis with emetine can stimulate CSF-arrested eggs to complete meiosis in marine invertebrates such as mollusks and ascidians [77–79]. From these seminal studies it can be hypothesized that a short-lived protein has to be synthesized for the egg to remain cell cycle arrested. This short lived protein is the oocyte-specific kinase Mos [40,26,53].

Most of the translational control of mRNAs occurs at the rate-limiting initiation step of protein synthesis through the highly orchestrated action of translation initiation factors (IFs) (reviewed in [80]). The regulatory process can be achieved through repression of the translational machinery assembly at the cap (5′ end), and/or through polyadenylation at the 3′ end by cis-acting elements. During meiotic maturation, Mos is regulated through mRNA translational control via cis acting elements contained in the 3′ UTR (reviewed in [81]). In *Xenopus*, P100 (Pat1 in yeast) and Eg2 (Aurora) kinases phosphorylate the initiation factor CPEB to induce Mos translation during the oocyte maturation ([82,83]). In the starfish *A. aranciacus*, Mos synthesis seems to be regulated by a mTOR-independent pathway [92]. However mechanisms of Mos synthesis and destruction are completely unknown in other marine invertebrate deuterostomes.Progression through meiosis relies on CPEB-regulated cyclin B synthesis [92]. CPEB must be phosphorylated by cdk1 to dissociate from the cap-binding protein eIF4E in order to promote cyclin B synthesis [93], Fig. 2.

After fertilization and release from CSF arrest, cyclin B synthesis is necessary for entry into first mitosis (Fig. 2). In sea urchin eggs the presence of the translational machinery toolkit, and the activation of protein synthesis triggered at fertilization have been described [84–86]. Firstly, in the unfertilized egg, the repressor 4E-BP is hypophosphorylated and bound to its partner eIF4E to inhibit global mRNA translation, including the cyclin mRNAs [87,88]. Fertilization enables (somehow) the activation of mTOR signaling pathway which induces the hyperphosphorylation of 4E-BP, and also its subsequent degradation [86,87] Fig. 2. This seemingly redundant mechanism of phosphorylation followed by destruction (i.e., in somatic cells, hyperphosphorylation of 4EBP is sufficient to block protein synthesis) might be due to the fact that the phosphorylation of 4E-BP is not enough for its dissociation from eIF4E in sea urchin fertilized eggs [89] and its destruction is thus necessary to de-repress protein synthesis. Another translational regulatory process in the sea urchin egg is the presence of the phosphorylated form of eIF2alpha (on the Ser51) in the unfertilized eggs (Fig. 2). Fertilization triggers its permanent dephosphorylation which is permissive for the activation of protein synthesis and entry into first mitosis [90].

Even though these studies provide an extensive description of the translational machinery present in eggs and its regulation during oocyte maturation, several important questions remain. For example, what is the link between Ca2+, MAPK and protein synthesis activation during fertilization? We hope that future studies performed on marine invertebrate deuterostomes will shed light into these important questions.

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