Development 138, 885-895 (2011) doi:10.1242/dev.057133

Mos limits the number of meiotic divisions in urochordate eggs

Rémi Dumollard[1,*,†], Mark Levasseur[2,*], Céline Hebras[1], Philippe Huitorel[1], Michael Carroll[1], Jean-Philippe Chambon[1] and Alex McDougall[1,†]

SUMMARY

Mos kinase is a universal mediator of oocyte meiotic maturation and is produced during oogenesis and destroyed after fertilization. The hallmark of maternal meiosis is that two successive M phases (meiosis I and II) drive two rounds of asymmetric cell division (ACD). However, how the egg limits the number of meioses to just two, thereby preventing gross aneuploidy, is poorly characterized. Here, in urochordate eggs, we show that loss of Mos/MAPK activity is necessary to prevent entry into meiosis III. Remarkably, maintaining the Mos/MAPK pathway active after fertilization at near physiological levels induces additional rounds of meiotic M phase (meiosis III, IV and V). During these additional rounds of meiosis, the spindle is positioned asymmetrically resulting in further rounds of ACD. In addition, inhibiting meiotic exit with Mos prevents pronuclear formation, cyclin A accumulation and maintains sperm-triggered Ca^{2+} oscillations, all of which are hallmarks of the meiotic cell cycle in ascidians. It will be interesting to determine whether Mos availability in mammals can also control the number of meioses as it does in the urochordates. Our results demonstrate the power of urochordate eggs as a model to dissect the egg-to-embryo transition.

KEY WORDS: Fertilization, MAPK, Asymmetric cell division, Cell cycle, Meiotic spindle, Urochordate, Ascidian

INTRODUCTION

Entry into and exit from mitosis is regulated by cyclin accumulation and destruction (Evans et al., 1983; for a review, see Hunt, 2004). A central tenet of somatic cell cycles is that directionality is provided by cyclin B destruction rendering exit from M phase irreversible (Potapova et al., 2006). The corollary of this is that only one M phase occurs per cycle. However, during meiosis, two M phases occur one after the other. Moreover, akin to mitotic exit, exit from meoisis I depends on cyclin B destruction in oocytes from diverse species: *C. elegans* (Furuta et al., 2000), ascidian (Levasseur and McDougall, 2000) and mouse (Herbert et al., 2003). This lack of irreversibility creates the potential problem of how the oocyte limits the number of M phases to just two during maternal meiosis. Interestingly, it has been known for many years that the premature activation of mouse oocytes causes them to enter meiosis III rather than interphase (Kubiak, 1989). The Mos/MAPK pathway is known to regulate both the cell cycle and asymmetric cell division in eggs (Kishimoto, 2003; Verlhac et al., 2000; Amiel et al., 2009). However, although it is well established in mammals and urochordates that a fall in MAPK activity precedes entry into interphase, it is not known whether it is the duration of Mos/MAPK activity that limits the number of meioses to just two.

Mos and its associated MAPK activity are elevated during maternal meiosis I and II throughout the Eumetazoa: Cnidaria (Amiel et al., 2009), Mollusc (Shibuya et al., 1992), Echiura (Gould and Stephano, 1999), Echinoderms (Tachibana et al., 1997), Urochordates (Russo et al., 1996; McDougall and Levasseur 1998), Vertebrates (Hashimoto et al., 1994; Verlhac et al., 1996). Although the role of Mos/MAPK pathway in CSF arrest is widespread (Sagata, 1989; Sagata, 1997; Masui, 2000; Yamamoto et al., 2008; Wu and Kornbluth, 2008), its role in other oocyte processes, such as asymmetric cell division (ACD), S phase inhibition and meiosis I to II transition, has either not been as well characterised [ACD (Verlhac et al., 2000; Amiel et al., 2009; Azoury et al., 2009)] or is less conserved [MI-to-MII transition (Furuno et al., 1994; Picard et al., 1996; Walter et al., 1997; Bitangcol et al., 1998; Tachibana et al., 2000; Verlhac et al., 2000; Dupre et al., 2002). Even less well studied is whether Mos/MAPK activity has to be switched off to prevent entry into meiosis III. Some evidence supporting this hypothesis has been found in mammalian oocytes. For example, maintaining MAPK active in mouse oocytes leads to the re-establishment MPF activity and prevents pronuclei formation after exit from meiosis II (Moos et al., 1995; Moos et al., 1996). However, it has not been tested whether keeping MAPK active in these eggs generates a third meiotic division, leading to extrusion of a third polar body (PB). As mouse oocytes can enter meiosis III (Kubiak, 1989) with elevated MAPK activity during the meiosis II to III transition (Verlhac et al., 1996), this suggests that the level of Mos/MAPK activity might be the determining factor limiting the number of meiosis to just two in eggs but this hypothesis has not been formally tested.

Ascidian and mammalian eggs share several common features. Ascidians are marine chordates (termed urochordates) positioned as a sister group of vertebrates (Delsuc et al., 2006). Ascidian eggs are arrested at metaphase I by CSF (Russo et al., 2009) in a similar manner to mammalian eggs that arrest at metaphase II (Shoji et al., 2006). As in mammals, fertilization in urochordates triggers a series of periodic Ca^{2+} waves that stimulate metaphase exit driven by cyclin B destruction and the loss of CDK1 activity (Levasseur and McDougall, 2000; Nixon et al., 2002). In both ascidian and

[1]Developmental Biology Unit UMR 7009, UMPC Univ. Paris 06 and Centre National de la Recherche (CNRS), Observatoire Océanologique, 06230 Villefranche-sur-Mer, France. [2]Institute of Cell and Molecular Bioscences, The Medical School, Framlington Place, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, UK.

*These authors contributed equally to this work
†Authors for correspondence (remi.dumollard@obs-vlfr.fr; alex-mc.dougall@obs-vlfr.fr)

Accepted 7 December 2010

mammalian eggs, sperm-triggered Ca^{2+} oscillations terminate minutes prior to meiotic exit (Stricker, 1999). Moreover, these sperm-triggered Ca^{2+} oscillations can be maintained by preventing meiotic exit. This positive-negative feedback cycle ensures that Ca^{2+} oscillations cease upon exit from the meiotic cell cycle (Dumollard et al., 2002). In order to dissect the role of Mos in CSF arrest from its role in the transition from meiosis II to interphase, we exploited the ascidian model as their eggs are arrested in metaphase I rather than metaphase II (as in mammals). Indeed, CSF is inactivated at fertilization during meiosis I in ascidians making it possible to study the meiosis II to interphase transition independent of CSF.

Remarkably, for a protein that has been studied for more than 20 years we would like to attribute one additional and central role to Mos in urochordates. We find that maintaining Mos and the downstream MAPK activity elevated after fertilization induces meiosis III, meiosis IV and even meiosis V, and also that each additional phase of meiosis is separated by a morphologically normal ACD. Mos kinase from three different taxa and mediating three different CSF arrests were tested [from the jellyfish *Clytia hemisphaerica* (G1 CSF), the ascidian *Ciona intestinalis* (Metaphase I CSF) and mouse Mos (Metaphase II CSF)] and all types of Mos had the same ability to produce supernumerary PBs in ascidian eggs. These observations prompt us to conclude that it is the duration of Mos/MAPK activity during fertilization of urochordate eggs that limits the number of meiotic M phases to precisely two.

MATERIALS AND METHODS

Biological material

Eggs from the ascidians *Phallusia mammillata* and *Ciona intestinalis* were harvested from animals obtained in Sète and kept in the laboratory in a tank of natural sea water at 16°C. Eggs from *Ascidiella aspersa* were harvested from Blyth, UK and kept in a tank of natural sea water at 16°C. Egg preparation and microinjection have been described previously (Dumollard and Sardet, 2001) [see detailed protocols by Sardet et al. (Sardet et al., 2010)]. All imaging experiments were performed at 19°C and the kinase assays at 21°C.

Molecular tools

Construction of Δ90-cyclin B::GFP has been described previously (Levasseur and McDougall, 2000; Levasseur and McDougall, 2003). Cyclin B Y170 was created by site-directed mutagenesis from full-length cyclin B [characterized by Levasseur and McDougall (Levasseur and McDougall, 2000)]. *Clytia hemispherica* mos1 has been cloned and described recently (Amiel et al., 2009), whereas *Mus musculus* Mos was a kind gift from Marie-Helene Verlhac (Université Pierre et Marie Curie, Paris, France). Ci-Mos (gene Id: KH.C2.835.v1.A.ND1-1), Ci-cyclin A (gene Id: KH.C2.821.v1.A.ND1-1), Ci-cyclin E (KH.S498.1.v2.A.SL1-1) and Ci-shugoshin (KH.C12.362.v1.A.SL1-1) were amplified from a *Ciona intestinalis* Gateway-compatible cDNA library using PCR. All constructs were made using pSPE3 and the Gateway cloning system (Invitrogen) unless otherwise stated [for a detailed protocol, see Sardet et al. (Sardet et al., 2010)]. For all fluorescent fusion protein constructs, we indicate the N or C terminal position of the fluorescent protein (FP) by the following convention: FP-protein X or protein X::FP.

Imaging of microtubules (to visualize spindles) was achieved by expression of microinjected mRNA coding for the microtubule binding domain of Map7 (also known as ensconsin) fused to Venus fluorescent protein while H2B::Rfp1 was used to follow DNA (see Prodon et al., 2010).

Synthetic mRNAs were prepared as concentrated solutions (>2 μg/μl) in distilled water and small aliquots were frozen at –80°C. Solutions were centrifuged for 15 minutes at 15,000 ***g*** prior to loading the needle in order to sediment particles that might block the injection needle. Fluorescence from fluorescent protein constructs was observed in an unfertilized egg a few hours after injection of concentrated mRNAs and less than one hour after injection in an embryo (two-cell stage).

Use of pharmacological inhibitors

Treatments with U0126 and nocodazole were done by bathing in sea water. UO126 was dissolved in DMSO and added to a final concentration of 1 μM for UO126 and 2 μM for nocodazole. Ionomycin, made up in ethanol, was added to eggs at 2 μM concentration for 4-5 minutes and then thoroughly washed out to allow for undisturbed PB extrusion.

BrdU incorporation

S-phase was determined by 5-Bromo-2-deoxyUridine (BrdU) incorporation as modified from Denker et al. (Denker et al., 2008). In brief, eggs or embryos were incubated in 1.5 mM BrdU in ASW for 2-4 hours followed by rapid rinsing in ASW without BrdU before fixation in 3.7% formaldehyde for 1 hour, rinsing, then treatment in 4 M HCl for at least 1 hour. After several rinsing steps, BrdU was detected by anti-BrdU antibodies and the appropriate fluorescent secondary antibodies, and observed by confocal microscopy on a Leica TCS SP2.

Time-lapse and fluorescence microscopy

Time-lapse imaging of Venus and Rfp1 constructs was performed on an Olympus IX70 inverted microscope set up for epifluorescence imaging. Sequential bright-field and fluorescence images were captured using a cooled CCD camera (Micromax, Sony Interline chip, Princeton Instruments, Trenton, NJ, USA) and data collected was analysed using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

Calcium imaging was performed by measuring the fluorescence of injected calcium green 1 dextran (10,000 MW, Invitrogen) as previously described (Dumollard and Sardet, 2001) [for detailed protocols, see Sardet et al. (Sardet et al., 2010)].

Imaging of eggs and embryos were analysed by MetaMorph software (Molecular Devices) and Image J (NIH, USA).

MBP kinase and histone H1 kinase measurements in *Phallusia mammillata* eggs

The MBP (Myelin Basic Protein) kinase activity and histone H1 kinase activity were assayed as described previously (McDougall and Levasseur, 1998). Samples of five oocytes were collected by first washing the oocytes through 1 M glycine (pH 8) three times to remove the sea water (this does not alter meiotic progression). The oocytes were then removed in a volume of 1 μl and transferred to 4 μl reaction buffer [25 mM HEPES, 80 mM β-glycerophosphate, 5 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 10 mg/ml leupeptin/pepstatin/aprotinin, 0.2 mM AEBSF, 1 mM benzamidine, 100 mM $NaVO_4$, 5 mM NaF (pH 7.2)]. At this point, the oocytes were snap-frozen in liquid nitrogen. After defrosting, samples were treated exactly as described previously (McDougall and Levasseur, 1998).

RESULTS

MAPK inactivation in an unfertilized egg promotes entry into interphase after one meiotic division

Mature ascidian eggs are arrested at metaphase I and display elevated MPF and MAPK activity (Russo et al., 2009; McDougall and Levasseur, 1998). We estimated MAPK and MPF activity in *Phallusia mammillata* eggs by measuring MBP and histone H1 kinase activity in fertilized and Ca^{2+}-activated eggs (Fig. 1). H1 kinase measurements show two peaks of MPF activity corresponding to the two meiotic divisions following fertilization (Fig. 1A). Activation of the egg with a Ca^{2+} pulse (4 minutes application of 2 μM ionomycin) also resulted in two peaks of MPF activity (see Fig. S1A in the supplementary material) as well as extrusion of two polar bodies (PBs) followed by meiotic exit and pronucleus (PN) formation (see Fig. S4 in the supplementary material) with similar kinetic to natural fertilization. MBP kinase assay indicates that MAPK activity is elevated before fertilization,

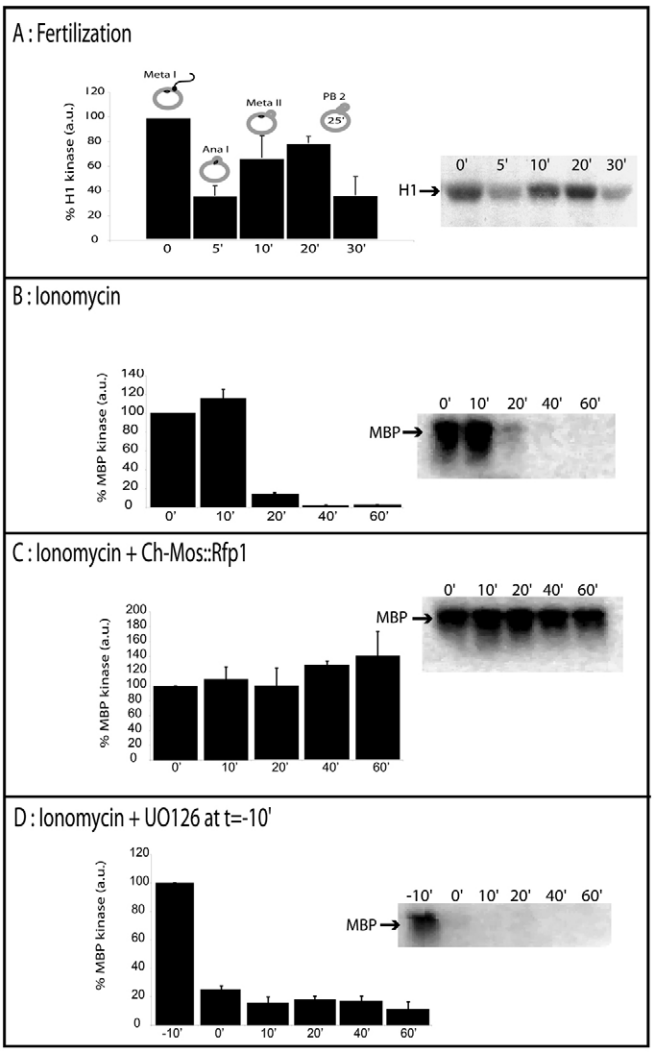

Fig. 1. Measuring and manipulating MAPK activity in *Phallusia mammillata* eggs. (**A**) MPF activity (estimated by histone H1 kinase assay) in *P. mammillata* eggs during fertilization. MPF is high in metaphase I arrested eggs (t=0′) then decreases just before PB1 extrusion (t=5′), while a second peak of MPF corresponding to meiosis II is observed (peak at 20′) before extrusion of the second polar body (PB2). Inset is an example of a gel that was analysed (five oocytes per lane, four kinase assays). (**B**) MAPK activity (estimated by MBP kinase assay) in *P. mammillata* eggs during Ca^{2+} activation (with ionomycin). MAPK is high in metaphase I arrested eggs and decreases 20 minutes after Ca^{2+} activation. Inset is an example of a gel that was analysed (five oocytes per lane, four kinase assays). (**C**) MAPK activity in *P. mammillata* eggs expressing Ch-Mos::Rfp1 during Ca^{2+} activation (with ionomycin). MAPK remains high for at least 1 hour. Inset is an example of a gel that was analysed (five oocytes per lane, four kinase assays). Unfertilized eggs were injected and Ch-Mos::Rfp1 was allowed to express for 15 hours before the start of the experiment. (**D**) MAPK activity in *P. mammillata* eggs treated with 1 μM UO126 10 minutes before Ca^{2+} activation with ionomycin (t=–10′). MAPK activity decreases within 10 minutes of UO126 treatment and remains low after Ca^{2+} activation. Inset is an example of a gel that was analysed (five oocytes per lane, four kinase assays). Data are mean ± s.e.m.

that it increases slightly during extrusion of the first PB (meiosis I to meiosis II transition) and then decreases 20 minutes after Ca^{2+} activation before exit from meiosis II (Fig. 1B, natural fertilization) (Russo et al., 1996; McDougall and Levasseur, 1998). Expression of Ch-Mos::Rfp1 (from jellyfish) in ascidian eggs is able to maintain MAPK activity indefinitely after egg activation (Fig. 1C). Expression of Mos from the ascidian *Ciona intestinalis* (Ci-Mos::Cherry) in unfertilized *Phallusia mammillata* eggs increased MAPK activity by 46% (Fig. 4D) and was also able to maintain MAPK activity after egg activation (data not shown). Incubation of ascidian eggs in the MEK inhibitor UO126 decreased MBP kinase activity in 10 minutes (Fig. 1D, Fig. 2B) confirming that MEK-induced MAPK activity is measured in our MBP kinase assay. These data demonstrate that UO126 rapidly suppresses MAPK activity and that, conversely, maintaining Mos levels in the egg is able to suppress MAPK downregulation usually observed after Ca^{2+} induced egg activation.

In order to assess the involvement of MAPK in metaphase I CSF we inactivated MAPK (using UO126) in unfertilized eggs and monitored egg activation in UO126-treated eggs (Fig. 2). Egg activation was monitored by measuring H1 kinase activity, cyclin B destruction as well as PB formation, PN formation and entry into S phase (Fig. 2A-C). Fig. 2A and Fig. S1C in the supplementary material show that, at around 32′ after incubation in 1 μM UO126, unfertilized *Phallusia mammillata* eggs extruded a PB that was sometimes reabsorbed (*n*=25 eggs). Unfertilized *Ciona intestinalis* eggs also extruded a PB after UO126 treatment (see Fig. S2A in the supplementary material). However, these PBs did not contain DNA (see Fig. S2A in the supplementary material) suggesting that proper chromosomes segregation did not occur. Nevertheless, such pseudo-PB formation was followed ~7′30″ later by formation of a PN (*n*=13, Fig. 2A and see Fig. S1C in the supplementary material). H1 kinase measurements showed that MPF declined at around 40 minutes after UO126 treatment, confirming exit from metaphase arrest (Fig. 2B). Furthermore, we consistently found that cyclin B was destroyed following inhibition of MAPK by UO126 treatment (Fig. 2C, *n*=7 eggs). Although PB formation was substantially delayed after U0126 treatment when compared with Ca^{2+} activated control eggs, PN formation occurred 7′30″ after PB extrusion in U0126-activated eggs, similar to the delay between PB2 and PN in controls (Fig. 2A; see Fig. S1C in the supplementary material). It should be noted that meiosis II was completely absent and that U0126-activated eggs entered interphase directly following extrusion of one PB.

Inhibition of the Mos/MAPK/p90rsk cascade in starfish oocytes can induce embryonic cleavages and parthenogenesis (Tachibana et al., 2000; Mori et al., 2006), but such parthenogenesis was never observed in ascidian eggs treated with UO126. However, we did notice cyclical deformations during the first hours of UO126 treatment with some deformations resembling pseudo-cleavages (see Movie 1 in the supplementary material). To assess for mitotic cell cycles in UO126-treated eggs, we monitored the cycles of accumulation of cyclin A during interphase and destruction during M phase. Ascidians possess one isoform of cyclin A. Cyclin A cannot accumulate during meiosis, which possesses only M phases, and can accumulate only during interphase. We thus expressed and imaged Ci-cyclin A::Venus in UO126-treated eggs (Fig. 2D). We first confirmed that Ci-cyclin A::Venus was destroyed during mitosis and synthesized during interphase in living ascidian embryos (see Fig. S3 in the supplementary material). Ci-cyclin A::Venus was found to be nuclear during interphase and accumulated until NEB, before being destroyed during the whole of mitosis (see Fig. S3 in the supplementary material), thus reproducing the known behaviour of cyclin A in somatic cells (Geley et al., 2001; Den Elzen and Pines, 2001).

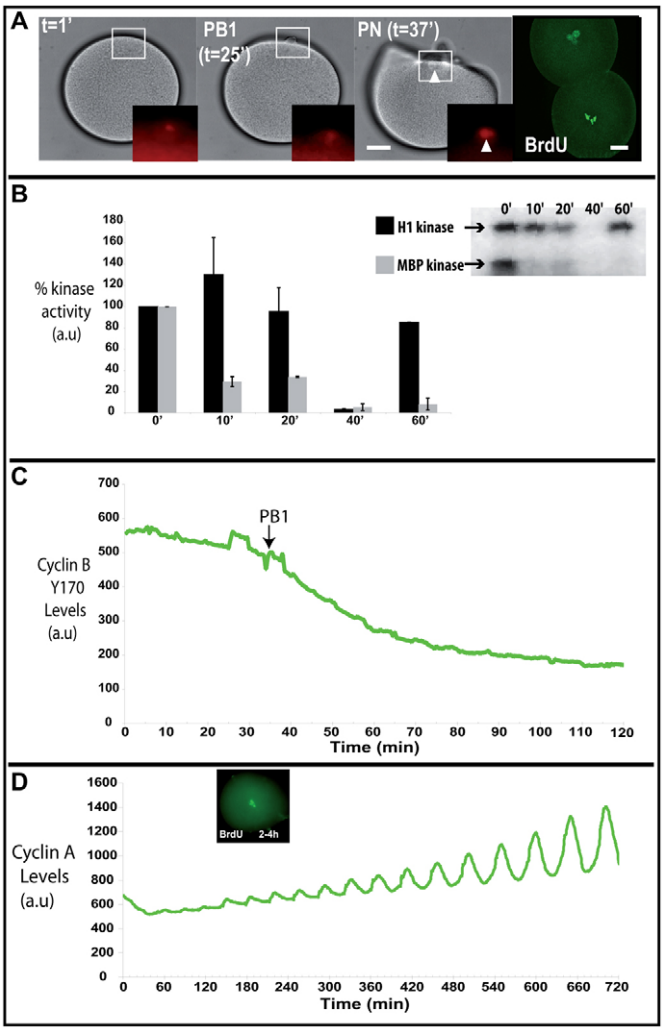

Fig. 2. The Mos/MAPK cascade mediates metaphase I CSF arrest in ascidian eggs. (**A**) Unfertilized egg treated with 1 μM UO126. Such eggs extrude one polar body (PB) and form a pronucleus (PN, arrowhead) that incorporates BrdU during S phase (BrdU image). DNA is shown in red from the H2B::Rfp1 image. Scale bars: 20 μm. (**B**) MBP (grey) and H1 (black) kinase activities in unfertilized eggs treated with 1 μM UO126 (at t=0′). Mean ± s.e.m. from three experiments. (**C**) The level of cyclin B/Y170::YFP expressed in an unfertilized *Phallusia* egg treated with 1 μM UO126 at t=0′. A polar body (PB) is extruded in this egg 35 minutes after UO126 addition. Unfertilized eggs were injected with mRNA encoding cyclin B/Y170::YFP and allowed to express for 15 hours before the start of the experiment. (**D**) Time course of Ci-cyclin A::Venus levels in an egg expressing Ci-cyclin A::Venus and treated with 1 μM UO126 at t=0′. After first degrading cyclin A upon meiotic exit, oscillations of cyclin A levels are observed corresponding to cycles of synthesis of cyclin A during interphase and destruction from NEB to telophase. Such eggs could incorporate BrdU (BrdU image) between 2 and 4 hours after UO126 addition, showing that, during this time, S phase had occurred. Unfertilized eggs were injected with mRNA encoding Ci-cyclin A::Venus and allowed to express for 15 hours before the start of the experiment.

Imaging of Ci-cyclin A::Venus consistently showed cycles of synthesis and destruction of cyclin A::Venus in UO126-treated eggs (Fig. 2D and see Movie 1 in the supplementary material; *n*=10 eggs). To confirm that DNA replication occurred repetitively in these eggs, we assessed BrdU incorporation during the first 2 hours of UO126 treatment (Fig. 2A) and between 2 and 4 hours after UO126 treatment (Fig. 2D) and found that these eggs indeed replicated DNA during both time windows. These observations demonstrate that MAPK inactivation alone is able to release CSF arrest, leading to exit from meiosis I immediately followed by entry into first interphase and subsequent mitotic cell cycles.

Both cyclin E/CDK2 and the SAC have also been implicated in mediating CSF arrest in eggs (Tunquist et al., 2002; Tunquist and Maller, 2003; Grimison et al., 2006). For example, studies in *Xenopus* egg extracts revealed that keeping cyclin E/CDK2 active can maintain M-phase after Ca^{2+} activation of CSF extracts (Tunquist et al., 2002). We thus tested for a potential role for cyclin E/CDK2 in CSF arrest in ascidians (see Fig. S4 in the supplementary material). Overexpression of cyclin E did not prevent PB formation but did inhibit PN formation (see Fig. S4B,D in the supplementary material). We reasoned that if cyclin E alone constitutes CSF then it should block egg activation induced by inactivation of MAPK. However, we found that inactivating MAPK in cyclin E-expressing eggs resulted in PB extrusion with the same kinetics as control eggs activated with U0126 (*n*=5, data not shown). This suggests that cyclin E/CDK2 may not be involved in metaphase I arrest in ascidians but confirmation of this will await knockdown of cyclin E in unfertilized eggs.

In order to assess whether the SAC is part of CSF-mediated arrest in ascidian eggs, we fertilized eggs in the presence of microtubule-depolymerising drugs (see Fig. S4 in the supplementary material). We found that although spindle disruption by nocodazole treatment did prevent PB extrusion it did not alter the timing of PN formation, suggesting that the SAC might not be functioning in ascidian eggs (see Fig. S4C,D in the supplementary material; *n*=18 eggs). However, these data do not rule out a role for SAC proteins in Meta-I CSF, which could be determined by a knockdown approach of candidate proteins. However, these data highlight a lack of feedback control monitoring progression of PB extrusion.

Together these data suggest that the Mos/MAPK cascade is the major regulator of CSF activity in urochordate eggs. Furthermore, the direct entry into interphase after one meiotic division in UO126-treated eggs indicates that MAPK is necessary for entry into meiosis II.

MAPK inactivation at fertilization prevents spindle formation during meiosis II but not MPF reactivation

Treatment of unfertilized egg with UO126 stimulates completion of one meiotic division before entry into first interphase, suggesting that the Mos/MAPK cascade promotes entry into meiosis II. However, when eggs are activated with UO126, meiotic completion requires around 30 minutes, whereas meiosis I completion takes only 7-9 minutes in Ca^{2+} activated (and fertilized) eggs (see Fig. S1C in the supplementary material). In order to confirm that Mos/MAPK promotes entry into meiosis II after fertilization, we treated eggs with UO126 and then activated the eggs with Ca^{2+} (see Fig. S1B,C in the supplementary material). Such eggs extruded a first PB at the normal time (~8 minutes after Ca^{2+} activation, see Fig. S1C in the supplementary material) but the second PB was not consistently observed (data not shown) and the metaphase II spindle could not form (see Fig. S1B in the supplementary material). PN formation occurred at the normal time (~31 minutes after Ca^{2+} activation, see Fig. S1C in the supplementary material). The late occurrence of PN formation indicates that meiosis II did occur. Measurements of H1

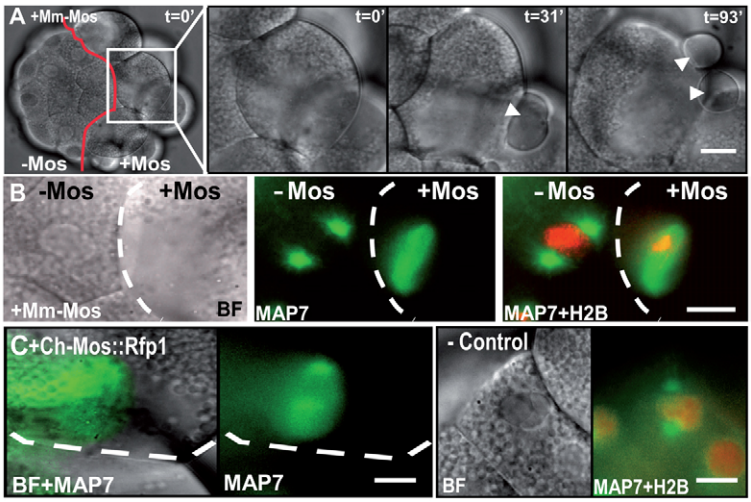

Fig. 3. Mos expression in an embryonic blastomere induces metaphase arrest. (**A**) Ascidian embryo injected at the two-cell stage with mRNA encoding Mm-Mos. One of the blastomeres of the embryo half expressing Mos (right hand side, +Mos) did not stop cleaving immediately but rather cleaved asymmetrically, generating small polar body-like cells (arrowheads at t=31′ and 93′). Scale bar: 20 μm. (**B**) Image of an embryo expressing MAP7::GFP and H2B::Rfp1 (expressing for 15 hours before fertilization) and injected with Mm-Mos at the two-cell stage. One blastomere is from the uninjected side (–Mos), while the other blastomere is from the injected embryo half (+Mos) (left image, BF; middle image, MAP7::GFP only; right image, overlay MAP7::GFP and H2B::Rfp1). The arrested blastomere (+Mos) contains a metaphase spindle contacting the plasma membrane (dotted line), whereas the uninjected blastomere is in prophase. Scale bar: 15 μm. (**C**) Images of a Ch-Mos::Rfp1-expressing blastomere (left image, Mos expressing blastomere; right image, blastomere from the control uninjected embryo half). The arrested blastomere (+Mos) contains a metaphase spindle contacting the plasma membrane (dotted line). By contrast, the control blastomere is in prophase. Scale bars: 15 μm.

kinase activity showed Ca^{2+} activation in an egg devoid of MAPK activity (treated with UO126 10 minutes before ionomycin treatment, Fig. 1D) also resulted in two peaks of MPF activity (see Fig. S1B in the supplementary material), suggesting that MPF could reactivate after PB1 extrusion without MAPK activity (20′ after Ca^{2+} activation, see Fig. S1B in the supplementary material). These observations indicate that MAPK activity is not necessary to drive entry into meiosis II during natural fertilization, even though MAPK activity is necessary for the formation of the metaphase II spindle (and hence extrusion of PB2).

Mos expression in embryonic blastomeres causes metaphase arrest

In order to confirm the involvement of the Mos/MAPK pathway in CSF activity, we expressed Mos in embryonic blastomeres by injecting capped RNAs coding for Mos kinase in one blastomere of two-cell stage embryos (see Fig. S2B in the supplementary material; Fig. 3). Injection of either jellyfish Ch-mos, mouse Mm-mos or ascidian Ci-mos cRNAs into one blastomere of a two-cell embryo all resulted in early cleavage arrest giving rise to large round blastomeres (see Fig. S2B in the supplementary material). Strikingly, we noticed that before reaching cleavage arrest (and sufficient accumulation of Mos protein expressed from injected capped RNA), Mos-expressing blastomeres underwent rounds of ACD reflected by the formation of PB-like cells in the blastomeres expressing Mos (arrowheads in Fig. 3A). Mos-expressing blastomeres eventually ceased cleaving and closer examination of blastomeres showed that they arrested in mitotic metaphase as seen by the sustained presence of a metaphase spindle (Mm-mos, n=5 embryos, Fig. 3B; Ch-mos, n=3 embryos, Fig. 3C). Remarkably, we noticed that the mitotic spindle in Mos-expressing blastomeres always had one pole subjacent to the plasma membrane suggestive of spindle pole anchoring to the cell cortex (Fig. 3B,C).

Together, these observations further suggest that the Mos/MAPK pathway mediates metaphase I CSF arrest in ascidians. They also indicate that, although a sufficient level of Mos is necessary to impose metaphase arrest in the embryo, a sub-threshold level of Mos promotes meiotic-like ACD over mitotic cell division.

Mos expression maintains the fertilized ascidian egg in a meiotic state

After establishing the role of the Mos/MAPK pathway in metaphase I CSF in urochordates, we sought to characterize other roles of Mos/MAPK during ascidian meiosis. We observed that inactivating MAPK in an unfertilized egg triggers meiotic exit after one PB (Fig. 2). Furthermore inactivating MAPK in a fertilized egg impairs formation of meiosis II spindle (see Fig. S1 in the supplementary material). These observations indicate that an active Mos/MAPK pathway may promote the meiotic phenotype of the mature egg (i.e. high MPF, small peripheral meiotic spindle). We thus tested whether maintaining the Mos/MAPK pathway active in fertilized eggs was able to inhibit Ca^{2+} triggered meiotic exit.

We used several criteria to define the meiotic state of the egg: (1) inhibition of pronuclear envelope formation after extrusion of PB2; (2) persistence of sperm-triggered Ca^{2+} oscillations after PB2 formation; (3) formation of a small meiotic spindle subjacent to the cortex after PB2 formation; (4) inhibition of cyclin A accumulation during interphase of the mitotic cell cycles observed after Ca^{2+} activation of the egg. We found that Mos-expressing fertilized eggs do not form pronuclei after completion of meiosis II. Although Mos expression did not affect the timing of PB1 and PB2 (Fig. 4A, t=32 min; see Fig. S5 in the supplementary material), no pronuclei could be observed in Mos-expressing eggs (boxed in red) at the time control eggs completed pronuclear migration (52 min post-fertilization, boxed in blue in Fig. 4A; see also Movie 2 in the supplementary material). Even though Ch-Mos blocks jellyfish

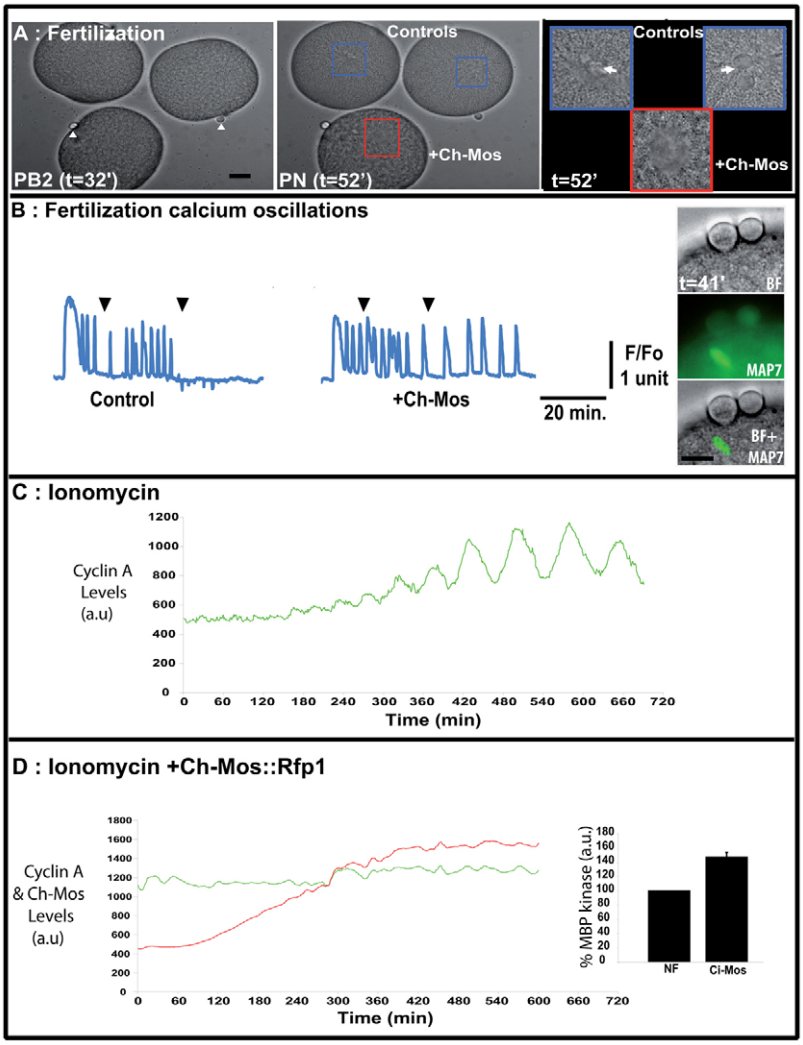

Fig. 4. The Mos/MAPK cascade maintains the meiotic cell cycle in ascidians. (**A**) Images showing one ascidian zygote expressing Ch-Mos::Rfp1 for 3 hours before fertilization (bottom) and two control uninjected zygotes (top). The three eggs have been fertilized simultaneously and extruded PB1 (not shown) and PB2 simultaneously (arrowheads at 32′). The blue insets show an enlarged view of the pronuclei (white arrows) prior to fusion 52′ after fertilization in control zygotes whereas no PN is visible in the Mos-expressing zygote (red box and inset). Scale bar: 30 μm. See Movie 2 in the supplementary material. (**B**) Sperm-triggered Ca^{2+} oscillations in a control egg (left graph) and in Ch-Mos::Rfp1-expressing egg (4 hours expression, right graph). The sperm triggered Ca^{2+} oscillations last for 30′ in control eggs, whereas they last for more than 70′ in Mos-expressing eggs (*n*=7 eggs). Black arrowheads indicate PB extrusion. Inset, egg expressing Ch-Mos::Rfp1 (for 3 hours) and MAP7::GFP (for 15 hours, to monitor the meiotic spindle) displaying a metaphase III spindle after extrusion of two PBs. (**C**) Time course of Ci-cyclin A::Venus levels in an egg expressing Ci-cyclin A::Venus (for 15 hours) and treated with 2 μM ionomycin at t=0′. Oscillations of cyclin A levels are observed, indicating that a mitotic cell cycle oscillator has been triggered by the initial Ca^{2+} increase. (**D**) Time course of Ci-cyclin A::Venus levels (green graph) in an egg expressing Ci-cyclin A::Venus (for 15 hours) and Ch-Mos::Rfp1 (for 4 hours, red graph), and treated with 2 μM ionomycin at t=0′. Neither accumulation nor oscillations of cyclin A levels are observed, indicating that such eggs could not enter interphase nor cycle, even though Ca^{2+} activation stimulated the egg (seen as an increase in Ch-Mos::Rfp1 reflecting the increase in protein translation accompanying egg activation). Graph shows that expression of Ci-Mos::Cherry in an unfertilized egg (for 15 hours) increases MBP kinase activity by 46% (*n*=4 experiments using five eggs each). Data are mean ± s.e.m.

eggs at G1 phase and Mm-Mos and Ci-Mos block eggs of their respective species at metaphase, the three different Mos proteins all inhibited PN formation in ascidian eggs (Fig. 4A; see Fig. S5 in the supplementary material).

The second criterion is based on the observation that sperm-triggered Ca^{2+} oscillations are associated with the meiotic cell cycle and that they terminate at the time the second PB is extruded ~7 minutes before the pronuclei form (McDougall and Levasseur, 1998). Moreover, blocking meiotic exit with a non-degradable form of cyclin B maintains the sperm-triggered Ca^{2+} oscillations indefinitely (Levasseur and McDougall, 2000). Strikingly, Mos overexpression also maintained sperm-triggered Ca^{2+} oscillations after extrusion of two PBs (Fig. 4B, *n*=7 eggs). Remarkably, we could observe in these eggs a small peripheral meiotic spindle after PB2 extrusion (inset in Fig. 4B), suggesting that maintaining Mos levels after fertilization induced entry into meiosis III.

Finally, we monitored cyclin A levels to further determine whether Mos maintained a meiotic-like phenotype. Activation of cyclin A::Venus-expressing ascidian eggs with ionomycin provoked extrusion of two PBs followed by PN formation (see Fig. S4 in the supplementary material) and cycles of accumulation and destruction of cyclin A::Venus indicative of mitotic cell cycles (Fig. 4C, *n*=5 eggs). Even though these Ca^{2+} activated eggs never cleaved, they proceeded through S phase, as monitored by BrdU incorporation into the egg DNA (see Fig. S4A in the supplementary material). Thus, Ca^{2+} activation of the egg is sufficient to stimulate exit from meiosis and to trigger a mitotic cell cycle oscillator driving successive S and M phases. We assessed the impact of Mos on this cell cycle oscillator by activating eggs expressing both cyclin A::Venus and Ch-Mos::Rfp1 with ionomycin (Fig. 4D). High Mos expression (overnight expression in the unfertilized egg) increased MAPK activity by only 46% (graph in Fig. 4D). In such eggs we could not measure any significant increase in cyclin A::Venus levels (Fig. 4D, *n*=7 eggs), even though they deformed, extruded two PBs and stimulated protein synthesis (seen as an increase in Ch-Mos::Rfp1 levels, red graph in Fig. 4D). The lack of cyclin A accumulation in the presence of Mos is indicative of a lack of mitotic cycles comprising interphases. Overall, these data show that Mos prevents the egg-to-embryo transition and maintains the fertilized egg in a meiosis-like state by inhibiting the triggering of a mitotic cell cycle oscillator. Strikingly Mos persistence after fertilization seems to promote entry into metaphase III.

Mos expression in fertilized eggs drives supernumerary rounds of meiotic divisions

We have shown so far that maintaining MAPK active in fertilized eggs can induce entry into metaphase III, as found previously in mouse eggs (Moos et al., 1995; Moos et al., 1996). However, we

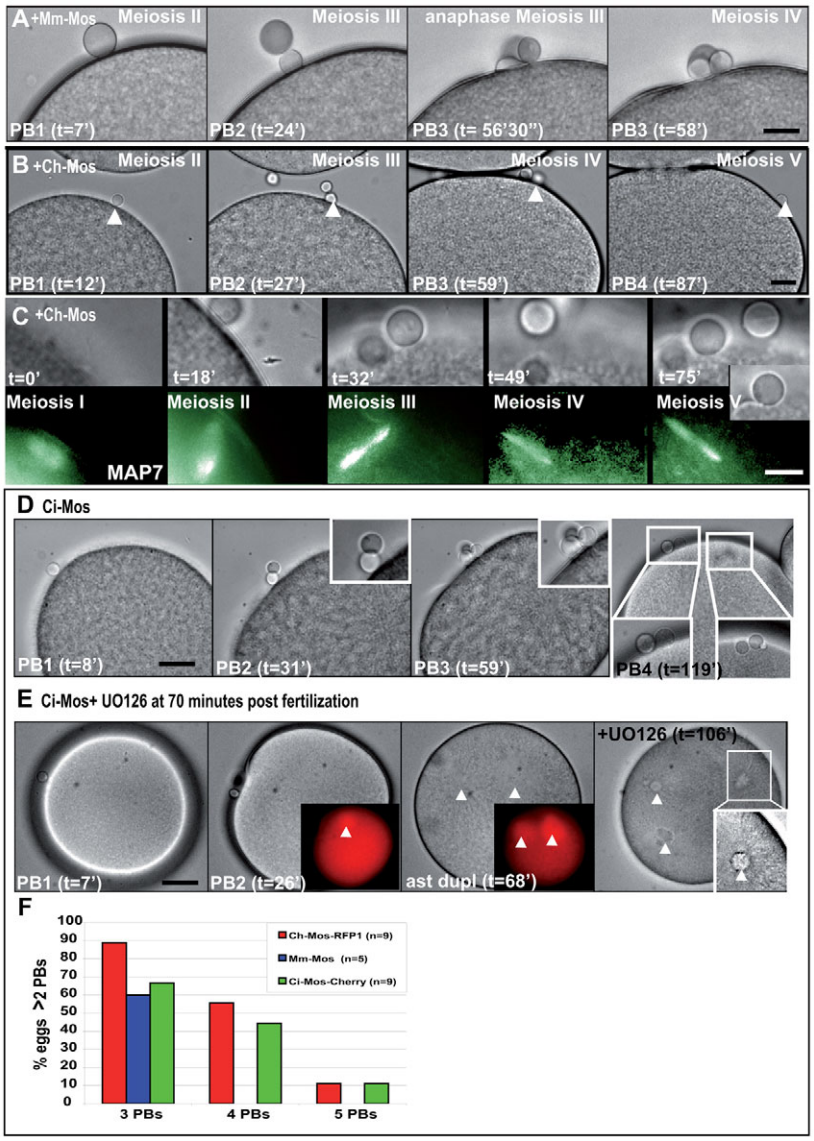

Fig. 5. Maintaining Mos/MAPK pathway activity in the fertilized ascidian egg induces supernumerary PBs. (**A**) Fertilized egg expressing Mm-Mos (for 12 hours) extruding three polar bodies (PB1 at 7′, PB2 at 24′ and PB3 at 56′30″) and hence undergoing meiosis I, II, III and IV. Scale bar: 10 μm. (**B**) Fertilized egg expressing Ch-Mos::Rfp1 (for 4 hours) extruding four polar bodies (arrowheads; PB1 at 12′, PB2 at 27′, PB3 at 59′ and PB4 at 87′) and hence undergoing meiosis I, II, III, IV and V. Scale bar: 20 μm. (**C**) Fertilized egg expressing Ch-Mos::Rfp1 (for 4 hours) and MAP7::GFP (for 15 hours). At t=0′, images of the meiosis I spindle; at t=18′, images of PB1 and meiosis II spindle; at t=32′, images of PB2 and meiosis III spindle; at t=49′, images of PB3 and meiosis IV spindle; at t=75′, images of PB4 and meiosis V spindle. Scale bar: 10 μm. (**D**) Fertilized egg expressing Ci-Mos::Cherry (for 4 hours) extruding four PBs. The first PB is extruded at 8′, the second PB at 31′, PB3 is extruded at 59′ and four PBs are visible at 119′ post-fertilization. Scale bar: 20 μm. (**E**) Fertilized egg expressing Ci-Mos::Cherry (for 15 hours, red insets) extruding two PBs (t=7′ and t=26′) and failing to produce a PN at 68′ post-fertilization [even though sperm aster (arrowheads) duplication occurred]. Perfusion of 5 μM UO126 at 70′ post fertilization triggered nuclei formation (white arrowheads and inset) around 30′ after UO126 perfusion (*n*=4 oocytes). Scale bar: 20 μm. (**F**) The number of PBs formed in fertilized eggs expressing the three different Mos constructions. Control eggs all produced only two PBs (not shown), whereas Mos-expressing eggs (expression between 4 and 15 hours) were observed to extrude either three, four or five PBs.

were surprised to find that longer time-lapse imaging of such eggs revealed that they extruded more than two PBs (Fig. 5 and see Movie 3 in the supplementary material). Fig. 5A-C shows fertilized ascidian eggs expressing either Mm-mos or Ch-mos, which were not blocked at metaphase III but extruded three PBs (Fig. 5A) while other eggs extruded four PBs (Fig. 5B,C) and sometimes even five PBs (Fig. 5F). This indicates that Mos expressing eggs are undergoing several additional rounds of meiosis (which we call meiosis III, IV and V) following completion of meiosis II. Exogenous Mos from either mouse or jellyfish or ascidian (Ci-Mos) all had this effect in *Phallusia mammillata* eggs (Fig. 5A-D). This striking effect was not limited to *Phallusia* eggs, as Ci-Mos could also induce more than two PBs in *Ascidiella aspersa* eggs (*n*=5 eggs, four PBs in the example shown in Fig. S6 in the supplementary material). We did not try this experiment in *Ciona intestinalis* eggs, as they have a low translation efficiency before fertilization (Prodon et al., 2010). None of the Mos constructs affected the timing of meiotic resumption as Mos-expressing eggs extruded the first two PBs with normal kinetics (*Mm-mos*, Fig. 5A, *n*=4 eggs; *Ch-mos*, Fig. 5B,C, *n*=14 eggs; Ci-mos, Fig. 5D, *n*=11 eggs, see Fig. S5 in the supplementary material). As the generation of supernumerary PBs requires a peripheral spindle to be present, we imaged microtubules in eggs overexpressing Mos. During meiosis III, IV and V, a peripheral meiotic spindle reformed after each PB extrusion and these spindles became thinner across their midsection with each round of meiosis (Fig. 5C, *n*=4 eggs). On one occasion, we observed that the meiotic spindle was eventually captured by the sperm aster, thereby preventing further formation of PBs (see Movie 4 in the supplementary material). Together, these observations demonstrate that maintaining Mos/MAPK cascade active in the fertilized egg is sufficient to stimulate entry into meiosis III, meiosis IV and even meiosis V. We performed the rescue experiment by treating Ci-Mos-expressing eggs that had been fertilized and had entered meiosis III with U0126 (i.e. 70 minutes after fertilization, Fig. 5E). Strikingly, we found that nuclei could be observed in such eggs around 30 minutes after perfusion of 5 μM UO126 (Fig. 5E, *n*=3 eggs) indicating that the ability of Mos to maintain a meiotic cell cycle is mediated entirely by MAPK

Overall these data show that an active Mos/MAPK pathway promotes a cortically located metaphase spindle in the fertilized egg, thereby ensuring spatial aspects of ACD during PB extrusion. Furthermore, the Mos/MAPK pathway must be downregulated after fertilization to limit the number of meioses to just two.

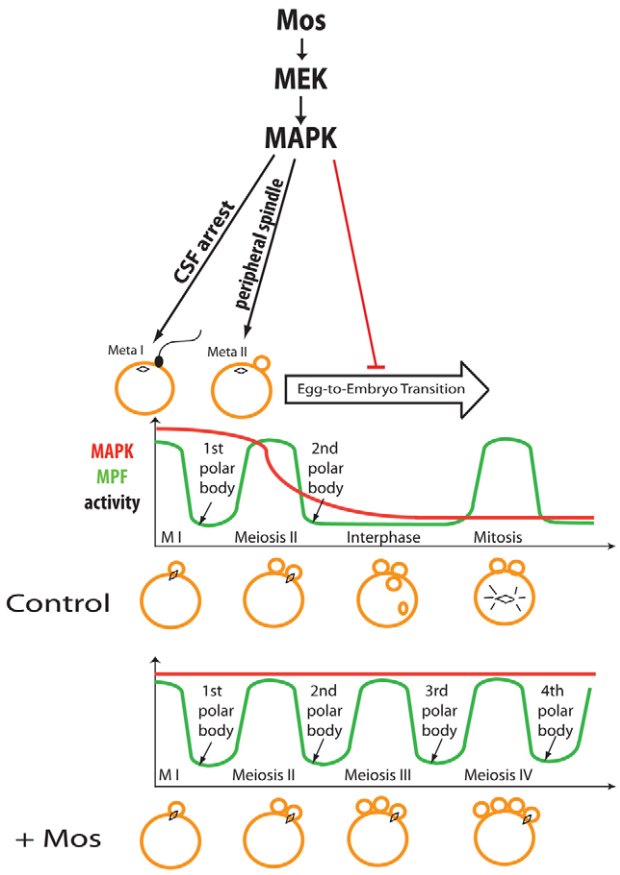

Fig. 6. Multiple roles for the Mos/MAPK cascade during the egg-to-embryo transition in urochordates. The Mos/MEK/MAPK cascade mediates metaphase I CSF arrest, promotes formation of a peripheral spindle and inhibits entry into mitotic cell cycles. Control: the levels of MPF (green) and MAPK (red) activities during meiosis completion and entry into mitotic cell cycles. MPF is inactivated upon meiosis I completion but re-activates rapidly upon entry into meiosis II. Then MPF decreases again upon meiosis II completion and remains low during the subsequent interphase before reactivating upon entry into first mitosis. MAPK remains elevated for 20 minutes after fertilization and thus decreases before meiosis II completion to allow for entry into first interphase. +Mos: in an eggs expressing Mos, MAPK activity remains elevated at near physiological levels and we surmise that MPF reactivates rapidly after each meiotic division, giving rise to supernumerary meioses.

DISCUSSION

By examining the role played by the Mos/MAPK pathway in metaphase I CSF arrest in urochordate eggs, we exposed the mechanism that limits the number of meioses in these eggs to two. Remarkably, by keeping the Mos/MAPK pathway active following fertilization at near physiological levels, the zygote continued to cycle in and out of meiosis. Specifically, these fertilized eggs entered meiosis III, formed a PB then entered meiosis IV and formed another PB and even occasionally entered meiosis V and formed a fifth PB (Fig. 6). We define these extra M phases as extra rounds of meiosis rather than mitosis by the following criteria: (1) a small anastral meiotic spindle forms subjacent to the cortex that drives rounds of PB formation during asymmetric cell division rather than a central mitotic spindle with large asters that drives symmetric cell division; (2) interphase-dependant cyclin A accumulation is abolished; (3) sperm-triggered calcium oscillations are maintained; and (4) nuclear envelope formation does not occur after PB formation. Therefore the Mos/MAPK pathway supports all aspects of meiotic asymmetric cell division in the egg and downregulation of the Mos/MAPK pathway is necessary to limit the number of meiotic M phases to two in fertilized urochordate eggs.

Mos/MAPK activity limits the number of meioses in the egg

A central tenet of mitosis is the irreversibility of mitotic exit. For example, in somatic cells a dramatic mechanism based on cyclin B destruction ensures that mitotic exit is irreversible and is followed by interphase (Potapova et al., 2006). This is not the case for meiosis. During maternal meiosis even though cyclin B destruction ensures exit from meiosis I in both urochordates and vertebrates oocytes, MPF activity comes back to drive entry into meiosis II (Levasseur and McDougall, 2000; Dupre et al., 2002; Herbert et al., 2003; Madgwick et al., 2006; Tang et al., 2008). This is probably due to the precocious inhibition of APC/C after anaphase I thereby allowing for early accumulation of cyclin B during interkinesis (Iwabuchi et al., 2000; Madgwick et al., 2006; Tang et al., 2008). The fact that meiosis I can be followed immediately by meiosis II makes it unclear how eggs avoid entering meiosis III. The Mos/MAPK pathway is a good candidate for regulating the number of meioses as it is a major regulator of the meiotic cell cycle (Kishimoto, 2003). However, this is not a universal mechanism because in both jellyfish and starfish the Mos/MAPK cascade is active during meiosis I and II and crucially remains active as the eggs exit meiosis II and enter G1 [jellyfish (Amiel et al., 2009), G1 or G2 in starfish (Fisher et al., 1998; Mori et al., 2006; Hara et al., 2009)]. Moreover, in *Xenopus* overexpression of Mos does not prevent meiotic exit and instead increases the duration of G2 in fertilized eggs (Murakami et al., 1999) or blocks activated CSF extracts in G2 phase (Bitangcol et al., 1998; Walter et al., 1997; Murakami and Vande Woode, 1998; Abrieu et al., 1997). However, in sharp contrast to *Xenopus*, maintaining MAPK activity in fertilized mouse eggs does inhibit pronucleus formation (Moos et al., 1995) but it is not know whether supernumerary meioses are induced.

Here, we find that maintaining Mos/MAPK activity in urochordate eggs not only inhibits entry into interphase but is also sufficient to generate supernumerary meioses. This is consistent with Mos-induced stabilization of MPF (Tachibana et al., 2000; Dupre et al., 2002; Kishimoto, 2003; Tang et al., 2008) and causes re-entry into additional rounds of meiotic M phase in urochordates (Fig. 6). The loss of the Mos/MAPK activity is necessary for preventing entry into further rounds of meiosis. However, this does not explain exit from these additional rounds of meiosis. As Mos also maintains sperm-triggered Ca^{2+} oscillations, we postulate that these Ca^{2+} increases drive exit from meiosis III, IV and V as they do at fertilization (McDougall and Sardet, 1995). Importantly, both low (2-3 hours expression) and high (15-20 hours expression) levels of Mos had the same effect in that all Mos-expressing eggs that were subsequently fertilized entered then exited meiosis III (data not shown).

Intriguingly, although MAPK activity drives entry into supernumerary meiosis (Fig. 5), Mos/MAPK activity was not found to be necessary for MPF reactivation after PB1 extrusion (see Fig. S1 in the supplementary material). This discrepancy might be due to a time delay between MAPK inactivation and MPF inactivation (Fig. 2). In fact, we observed that even though MAPK

is inactivated within 10 minutes of UO126 treatment (Fig. 1), MPF declines and cyclin B is destabilized only 30 minutes after UO126 treatment (Fig. 2). This suggests that MAPK stabilizes cyclin B (and MPF) indirectly and that the MAPK target inhibiting the APC/C remains active for up to 20 minutes after MAPK inactivation. For these reasons, we cannot exclude a role for a MAPK-dependent mechanism for entry into meiosis II in urochordates. By contrast, we demonstrate here that MAPK is required for formation of the metaphase II spindle.

Finally, the ability to form multiple PBs suggests that bipolar meiotic spindles in *Phallusia* (and *Ascidiella*) eggs can form independently of centrioles, as is the case in mouse oocytes. In mouse oocytes, meiotic spindles will form around chromosomes and several meiotic spindles can form in the same metaphase II-arrested oocyte (Maro et al., 1986). Even DNA-covered beads cause acentriolar bipolar meiotic spindles to form in mouse oocytes (Deng et al., 2006). However, in starfish oocytes, the metaphase I meiotic spindle contains two centrioles at each pole whereas the meiosis II spindle has one centriole at each pole (Tamura and Nemoto, 2001). Starfish oocytes lose the ability to form a bipolar meiotic spindle following extrusion of the second PB as only one centriole (which is non reproductive) remains in the egg (Tamura and Nemoto, 2001). We therefore surmise that a bipolar meiotic spindle can form in the ascidian without the need of centrioles similar to mouse oocytes. However, this should be confirmed by direct visualization of centrioles in ascidian eggs.

Mos and CSF arrest at metaphase I

CSF was originally described as a catalytic activity that is able to block amphibian eggs at metaphase II and was demonstrated by the cell cycle arrest caused by injecting egg cytoplasm into blastomeres at the two cell stage (Masui and Markert, 1971; for a review, see Masui, 2000; Kishimoto, 2003). Vertebrate CSF is mediated by an inhibitor of the APC/C called Emi2/Erp1 (for a review, see Wu and Kornbluth, 2008). Erp1 levels normally increase during meiosis II in *Xenopus* (Rauh et al., 2005; Tang et al., 2008) and in the mouse (Shoji et al., 2006), and although Erp1 levels decline following egg activation, Erp1 protein is still present up to the four-cell stage in mouse (Shoji et al., 2006) and up to the blastula stage in *Xenopus* (Nishiyama et al., 2007). Mos induces cleavage arrest in *Xenopus* embryo at the two-cell stage (Sagata, 1989) and this is probably due to residual Erp1. In amphibians Mos/MAPK/p90rsk phosphorylates Erp1 to maintain its inhibitory activity towards the APC/C (Nishiyama et al., 2007; Inoue et al., 2007).

Mos/MAPK activity mediates metaphase I CSF arrest in urochordates (Fig. 6). Our observations suggest that metaphase I CSF in urochordates is also an APC/C inhibitor activated by Mos/MAPK-dependant phosphorylation. Therefore, a possible conservation between metaphase I CSF in urochordates and metaphase II CSF in vertebrates might exist. However, although the identity of the ascidian APC/C inhibitor is not known, it is distinct from Emi2/Erp1, which is not present in invertebrate genomes, including urochordates (Russo et al., 2009; Fernandez-Guerra et al., 2006; Yamamoto et al., 2008). Further studies are thus needed to identify metaphase I CSF in invertebrate eggs. Once such a protein is found in urochordates, it will be possible to test the hypothesis that entry into meiosis II and determination of the number of meioses is due to re-establishment of CSF after meiotic exit and that re-establishment of CSF must be circumvented to enter the first mitotic cell cycle

Uncoupling the role of Mos in CSF and ACD in ascidian embryos

Another aspect of our findings is that Mos controls meiotic events occurring after fertilization and so meiotic processes distinct from CSF-induced cell cycle arrest such as asymmetric cell division (ACD). This additional role of Mos in ACD is probably ancestral and may have originated with anisogamy. As egg size increased relative to the sperm size in the Eumetazoa, this created the need to conserve oocyte cytoplasm during both meiotic divisions. Indeed, most species display oocytes that form small PBs, which minimizes loss of oocyte cytoplasm during both meiotic divisions. Some insects have even dispensed with cytokinesis altogether during meiosis and retain all four sets of haploid chromatids in their cytoplasm (*Drosophila* and Sawfly). Interestingly, Mos is not required for meiotic progression in *Drosophila* (Ivanovska et al., 2004). Nevertheless, for the majority of Eumetazoan species, Mos/MAPK activity correlates with PB extrusion during the two phases of ACD. An interesting example of how the Mos/MAPK pathway correlates with meiotic cell divisions rather than with CSF cell cycle arrest is found in some species of lophotrochozoa (e.g. some molluscs and Echiuran worms). For example, oocytes from some mollusc and Echiuran species are arrested at the GV stage before fertilization when MAPK activity is low (Shibuya et al., 1992; Gould and Stephano, 1999). It is only after fertilization that the MAPK activity increases correlating with ACD during meiosis I and II (Shibuya et al., 1992; Gould and Stephano, 1999). This correlation suggests that MAPK is required for processes associated with meiosis I and II rather than CSF in these species. Indeed, in ascidians (this study) and in many other species (jellyfish, echinoderm, amphibian and mammals) disrupting Mos function inhibits PB extrusion (Amiel et al., 2009; Tachibana et al., 2000; Bodart et al., 2005; Choi et al., 1996). For example, mouse oocytes lacking Mos display large PBs and a number of other defects (Verlhac et al., 2000). Premature downregulation of the Mos/MAPK pathway in ascidian eggs prevents metaphase II spindle formation, further confirming the major role of this pathway not only in cell cycle arrest but also in meiotic spindle function.

Thus, in addition to CSF, the Mos/MAPK cascade imposes the meiosis-like phenotype partly characterized by a small peripheral spindle necessary for PB formation during ACD.

Conclusion

In conclusion, we propose that the Mos/MAPK pathway maintains an egg phenotype typified by a small peripheral spindle, successive rounds of M phase without intervening interphases or cyclin A accumulation and ongoing Ca^{2+} oscillations. Because urochrodates are at the crossroad between the invertebrates and the vertebrates (Delsuc et al., 2006) it will be interesting to elucidate which processes are shared with either invertebrates or vertebrates.

Acknowledgements

We thank the ANR (08-BLAN-0136-02), ARC, AFM and ATIP for financial support. R.D. is supported by CNRS. We also w thank Evelyn Houliston and Sandra Chevallier for providing RNAs coding for Ch-Mos::rfp1, Marie-Helene Verlhac for providing the plasmid pRN3-Mm-Mos, and Christian Rouvière for technical support with image acquisition and image analysis.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.057133/-/DC1

References

Abrieu, A., Fisher, D., Simon, M. N., Dorée, M. and Picard, A. (1997). MAPK inactivation is required for the G2 to M-phase transition of the first mitotic cell cycle. *EMBO J.* **16**, 6407-6413.

Amiel, A., Leclère, L., Robert, L., Chevalier, S. and Houliston, E. (2009). Conserved functions for Mos in eumetazoan oocyte maturation revealed by studies in a cnidarian. *Curr. Biol.* **19**, 305-311.

Azoury, J., Verlhac, M.-H. and Dumont, J. (2009). Actin filaments: key players in the control of asymmetric division in mouse oocytes. *Biol. Cell* **101**, 69-76.

Bitangcol, J. C., Chau, A. S., Stadnick, E., Lohka, M. J., Dicken, B. and Shibuya, E. K. (1998). Activation of the p42 mitogen-activated protein kinase pathway inhibits Cdc2 activation and entry into M-phase in cycling Xenopus egg extracts. *Mol. Biol. Cell* **9**, 451-467.

Bodart, J. F., Baert, F. Y., Sellier, C., Duesbery, N. S., Flament, S. and Vilain, J. P. (2005). Differential roles of p39Mos-Xp42Mpk1 cascade proteins on Raf1 phosphorylation and spindle morphogenesis in Xenopus oocytes. *Dev. Biol.* **283**, 373-383.

Choi, T., Fukasawa, K., Zhou, R., Tessarollo, L., Borror, K., Resau, J. and Vande Woude, G. F. (1996). The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. *Proc. Natl. Acad. Sci. USA* **93**, 7032-7035.

Delsuc, F., Brinkmann, H., Chourrout, D. and Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965-968.

Den Elzen, N. and Pines, J. (2001). Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. *J. Cell Biol.* **153**, 121-136.

Deng, M., Suraneni, P., Schultz, R. M. and Li, R. (2006). The Ran GTPase mediates chromatin signaling to control cortical polarity during polar body extrusion in mouse oocytes. *Dev. Cell* **12**, 301-308.

Denker, E., Manuel, M., Leclère, L., Le Guyader, H. and Rabet, N. (2008). Ordered progression of nematogenesis from stem cells through differentiation stages in the tentacle bulb of Clytia hemisphaerica (Hydrozoa, Cnidaria). *Dev. Biol.* **315**, 99-11.

Dumollard, R. and Sardet, C. (2001). Three different calcium wave pacemakers in ascidian eggs. *J. Cell Sci.* **114**, 2471-2481.

Dumollard, R., Carroll, J., Dupont, G. and Sardet, C. (2002). Calcium wave pacemakers in eggs. *J. Cell Sci.* **115**, 3557-3564.

Dupre, A., Jessus, C., Ozon, R. and Haccard, O (2002). mos is not required for the initiation of meiotic maturation in Xenopus oocytes. *EMBO J.* **21**, 4026-4036.

Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D. and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**, 389-396.

Fernandez-Guerra, A., Aze, A., Morales, J., Mulner-Lorillon, O., Cosson, B., Cormier, P., Bradham, C., Adams, N., Robertson, A. J., Marzluff, W. F. et al. (2006). The genomic repertoire for cell cycle control and DNA metabolism in S. purpuratus. *Dev. Biol.* **300**, 238-251.

Fisher, D., Abrieu, A., Simon, M. N., Keyse, S., Vergé, V., Dorée, M. and Picard, A. (1998). MAP kinase inactivation is required only for G2-M phase transition in early embryogenesis cell cycles of the starfishes Marthasterias glacialis and *Astropecten aranciacus*. *Dev. Biol.* **202**, 1-13.

Furuno, N., Nishizawa, M., Okazaki, K., Tanaka, H., Iwashita, J., Nakajo, N., Ogawa, Y. and Sagata, N. (1994). Suppression of DNA replication via Mos function during meiotic divisions in Xenopus oocytes. *EMBO J.* **13**, 2399-2410.

Furuta, T., Tuck, S., Kirchner, J., Koch, B., Auty, R., Kitagawa, R., Rose, A. M. and Greenstein, D. (2000). EMB-30: an APC4 homologue required for metaphase-to-anaphase transitions during meiosis and mitosis in Caenorhabditis elegans. *Mol. Biol. Cell* **11**, 1401-1419.

Geley, S., Kramer, E., Gieffers, C., Gannon, J., Peters, J. M. and Hunt, T. (2001). Anaphase-promoting complex/cyclosome-dependent proteolysis of human Cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. *J. Cell Biol.* **153**, 137-148.

Gould, M. C. and Stephano, J. L. (1999). MAP kinase, meiosis, and sperm centrosome suppression in Urechis caupo. *Dev. Biol.* **216**, 348-358.

Grimison, B., Liu, J., Lewellyn, A. L. and Maller, J. L. (2006). Metaphase arrest by Cyclin E-Cdk2 requires the spindle-checkpoint kinase Mps1. *Curr. Biol.* **16**, 1968-1973.

Hara, M., Mori, M., Wada, T., Tachibana, K. and Kishimoto, T. (2009). Start of the embryonic cell cycle is dually locked in unfertilized starfish eggs. *Development* **136**, 1687-1696.

Hashimoto, N., Watanabe, N., Furuta, Y., Tamemoto, H., Sagata, N., Yokoyama, M., Okazaki, K., Nagayoshi, M., Takeda, N., Ikawa, Y. et al. (1994). Parthenogenetic activation of oocytes in c-mos-deficient mice. *Nature* **370**, 68-71.

Herbert, M., Levasseur, M., Homer, H., Yallop, K., Murdoch, A. and McDougall, A. (2003). Homologue disjunction in mouse oocytes requires proteolysis of securin and cyclin B1. *Nat. Cell Biol.* **5**, 1023-1025.

Hunt, T. (2004). The discovery of Cyclin. *Cell* **116**, S63-S64.

Inoue, D., Ohe, M., Kanemori, Y., Nobui, T. and Sagata, N. (2007). A direct link of the Mos-MAPK pathway to Erp1/Emi2 in meiotic arrest of Xenopus laevis eggs. *Nature* **446**, 1100-1104.

Ivanovska, I., Lee, E., Kwan, K. M., Fenger, D. D. and Orr-Weaver, T. L. (2004). The Drosophila MOS ortholog is not essential for meiosis. *Curr. Biol.* **14**, 75-80.

Iwabuchi, M., Ohsumi, K., Yamamoto, T. M., Sawada, W. and Kishimoto, T. (2000). Residual Cdc2 activity remaining at meiosis I exit is essential for meiotic M-M transition in Xenopus oocyte extracts. *EMBO J.* **19**, 4513-4523.

Kishimoto, T. (2003). Cell-cycle control during meiotic maturation. *Curr. Opin. Cell Biol.* **15**, 654-663.

Kubiak, J. Z. (1989). Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev. Biol.* **136**, 537-545.

Levasseur, M. and McDougall, A. (2000). Sperm-induced calcium oscillations at fertilisation in ascidians are controlled by Cyclin B1-dependent kinase activity. *Development* **127**, 631-641.

Levasseur, M. and McDougall, A. (2003). Inositol 1,4,5-trisphosphate (IP3) responsiveness is regulated in a meiotic cell cycle dependent manner: implications for fertilization induced calcium signaling. *Cell Cycle* **2**, 610-613.

Madgwick, S., Hansen, D. V., Levasseur, M., Jackson, P. K. and Jones, K. T. (2006). Mouse Emi2 is required to enter meiosis II by reestablishing Cyclin B1 during interkinesis. *J. Cell Biol.* **174**, 791-801.

Maro, B., Johnson, J. H., Webb, M. and Flach, G. (1986). Mechanism of polar body formation in the mouse oocyte: an interaction between the chromosomes, the cytoskeleton and the plasma memvbrane. *J. Embryol. Exp. Morphol.* **92**, 11-32.

Masui, Y. (2000). The elusive cytostatic factor in the animal egg. *Nat. Rev. Mol. Cell Biol.* **1**, 228-232.

Masui, Y. and Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177**, 129-145.

McDougall, A. and Sardet, C. (1995). Function and characteristics of repetitive calcium waves associated with meiosis. *Curr. Biol.* **5**, 318-328.

McDougall, A. and Levasseur, M. (1998). Sperm-triggered calcium oscillations during meiosis in ascidian oocytes first pause, restart, then stop: correlations with cell cycle kinase activity. *Development* **125**, 4451-4459.

Moos, J., Visconti, P. E., Moore, G. D., Schultz, R. M. and Kopf, G. S. (1995). Potential role of mitogen-activated protein kinase in pronuclear envelope assembly and disassembly following fertilization of mouse eggs. *Biol. Reprod.* **53**, 692-699.

Moos, J., Xu, Z., Schultz, R. M. and Kopf, G. S. (1996). Regulation of nuclear envelope assembly/disassembly by MAP kinase. *Dev. Biol.* **175**, 358-361.

Mori, M., Hara, M., Tachibana, K. and Kishimoto, T. (2006). p90rsk is required for G1 phase arrest in unfertilized starfish eggs. *Development* **133**, 1823-1830.

Murakami, M. S. and Vande Woude, G. F. (1998). Analysis of the early embryonic cell cycles of Xenopus; regulation of cell cycle length by Xe-wee1 and Mos. *Development* **125**, 237-248.

Murakami, M. S., Copeland, T. D. and Vande Woude, G. F. (1999). Mos positively regulates Xe-Wee1 to lengthen the first mitotic cell cycle of Xenopus. *Genes Dev.* **13**, 620-631.

Nishiyama, T., Ohsumi, K. and Kishimoto, T. (2007). Phosphorylation of Erp1 by p90rsk is required for cytostatic factor arrest in Xenopus laevis eggs. *Nature* **446**, 1096-1099.

Nixon, V. L., Levasseur, M., McDougall, A. and Jones, K. T. (2002). Ca(2+) oscillations promote APC/C-dependent cyclin B1 degradation during metaphase arrest and completion of meiosis in fertilizing mouse eggs. *Curr. Biol.* **12**, 746-750.

Picard, A., Galas, S., Peaucellier, G. and Dorée, M. (1996). Newly assembled Cyclin B-cdc2 kinase is required to suppress DNA replication between meiosis I and meiosis II in starfish oocytes. *EMBO J.* **15**, 3590-3598.

Potapova, T. A., Daum, J. R., Pittman, B. D., Hudson, J. R., Jones, T. N., Satinover, D. L., Stukenberg, P. T. and Gorbsky, G. J. (2006). The reversibility of mitotic exit in vertebrate cells. *Nature* **440**, 954-958.

Prodon, F., Chenevert, J., Hébras, C., Dumollard, R., Faure, E., Gonzalez-Garcia, J., Nishida, H., Sardet, C. and McDougall, A. (2010). Dual mechanism controls asymmetric spindle position in ascidian germ cell precursors. *Development* **137**, 2011-2021.

Rauh, N. R., Schmidt, A., Bormann, J., Nigg, E. A., Mayer, T. U. (2005). Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. *Nature* **437**, 1048-1052.

Russo, G. L., Kyozuka, K., Antonazzo, L., Tosti, E. and Dale, B. (1996). Maturation promoting factor in ascidian oocytes is regulated by different intracellular signals at meiosis I and II. *Development* **122**, 1995-2003.

Russo, G. L., Bilotto, S., Ciarcia, G. and Tosti, E. (2009). Phylogenetic conservation of cytostatic factor related genes in the ascidian Ciona intestinalis. *Gene* **429**, 104-111.

Sagata, N. (1989). The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* **342**, 512-518.

Sagata, N. (1997). What does Mos do in oocytes and somatic cells? *BioEssays* **19**, 13-21.

Sardet, C., McDougall, A., Yasuo, H., Chênevert, J., Prulière, G., Dumollard, R., Hudson, C., Hebras, C., Le Nguyen, N. and Paix, A. (2010). Embryological methods in ascidians: the Villefranche-sur-Mer protocols. *Methods Mol. Biol.* (in press).

Shibuya, E. K., Boulton, T. G., Cobb, M. H. and Ruderman, J. V. (1992). Activation of p42 MAP kinase and the release of oocytes from cell cycle arrest. *EMBO J.* **11**, 3963-3975.
Shoji, S., Yoshida, N., Amanai, M., Ohgishi, M., Fukui, T., Fujimoto, S., Nakano, Y., Kajikawa, E. and Perry, A. C. (2006). Mammalian Emi2 mediates cytostatic arrest and transduces the signal for meiotic exit via Cdc20. *EMBO J.* **25**, 834-845.
Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* **211**, 157-176.
Tachibana, K., Machida, T., Nomura, Y. and Kishimoto, T. (1997). MAP kinase links the fertilization signal transduction pathway to the G1/S-phase transition in starfish eggs. *EMBO J.* **16**, 4333-4339.
Tachibana, K., Tanaka, D., Isobe, T. and Kishimoto, T. (2000): c-mos forces the mitotic cell cycle to undergo meiosis II to produce haploid gametes. *Proc. Natl. Acad. Sci. USA* **97**, 14301-14306.
Tamura, M. and Nemoto, S. (2001). Reproductive maternal centrosomes are cast off into polar bodies during maturation division in starfish oocytes. *Exp. Cell Res.* **269**, 130-139.
Tang, W., Wu, J. Q., Guo, Y., Hansen, D. V., Perry, J. A., Freel, C. D., Nutt, L., Jackson, P. K. and Kornbluth, S. (2008). Cdc2 and Mos regulate Emi2 stability to promote the meiosis I-meiosis II transition. *Mol. Biol. Cell* **19**, 3536-3543.
Tunquist, B. J. and Maller, J. L. (2003). Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes Dev.* **17**, 683-710.
Tunquist, B. J., Schwab, M. S., Chen, L. G. and Maller, J. L. (2002). The spindle checkpoint kinase bub1 and Cyclin e/cdk2 both contribute to the establishment of meiotic metaphase arrest by cytostatic factor. *Curr. Biol.* **12**, 1027-1033.
Verlhac, M. H., Kubiak, J. Z., Weber, M., Geraud, G., Colledge, W. H., Evans, M. J. and Maro, B. (1996). Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. *Development* **122**, 815-822.
Verlhac, M. H., Lefebvre, C., Guillaud, P., Rassinier, P. and Maro, B. (2000). Asymmetric division in mouse oocytes: with or without Mos. *Curr. Biol.* **10**, 1303-1306.
Walter, S. A., Guadagno, T. M. and Ferrell, J. E. Jr. (1997). Induction of a G2-phase arrest in Xenopus egg extracts by activation of p42 mitogen-activated protein kinase. *Mol. Biol. Cell* **8**, 2157-2169.
Wu, J. Q. and Kornbluth, S. (2008). Across the meiotic divide – CSF activity in the post-Emi2/XErp1 era. *J. Cell Sci.* **121**, 3509-3514.
Yamamoto, D. S., Tachibana, K., Sumitani, M., Lee, J. M. and Hatakeyama, M. (2008). Involvement of Mos-MEK-MAPK pathway in cytostatic factor (CSF) arrest in eggs of the parthenogenetic insect, Athalia rosae. *Mech. Dev.* **125**, 996-1008.