Focus on Meiosis

Mammalian egg activation: from Ca^{2+} spiking to cell cycle progression

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Abstract

Mammalian eggs arrest at metaphase of the second meiotic division (MetII). Sperm break this arrest by inducing a series of Ca^{2+} spikes that last for several hours. During this time cell cycle resumption is induced, sister chromatids undergo anaphase and the second polar body is extruded. This is followed by decondensation of the chromatin and the formation of pronuclei. Ca^{2+} spiking is both the necessary and solely sufficient sperm signal to induce full egg activation. How MetII arrest is established, how the Ca^{2+} spiking is induced and how the signal is transduced into cell cycle resumption are the topics of this review. Although the roles of most components of the signal transduction pathway remain to be fully investigated, here I present a model in which a sperm-specific phospholipase C (PLC ζ) generates Ca^{2+} spikes to activate calmodulin-dependent protein kinase II and so switch on the Anaphase-Promoting Complex/Cyclosome (APC/C). APC/C activation leads to securin and cyclin B1 degradation and in so doing allows sister chromatids to be segregated and to decondense.

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Introduction

Of all the divisions our cells go through, the meiotic divisions of the gametes are unique. Only in meiosis do homologous chromosomes recombine, exchanging genetic information through chiasmata, to then be resolved by homologue segregation during the first meiotic division. Also uniquely, the first meiotic division is followed immediately by the second meiotic division in which sister chromatids are segregated (Fig. 1). The haploid gamete so generated therefore requires the haploid gamete of the opposite sex to restore the correct ploidy and in mammals at least, also fulfils the correct pattern of gene imprinting necessary for development to term.

This review concentrates on the mammalian egg and in particular the process of the second meiotic division. Mammalian eggs arrest most of their lives at the dictyate stage of prophase I and are stimulated to undergo the first meiotic division in the ovary under the influence of gonadotrophins. Just before ovulation and immediately following the first meiotic division, mammalian eggs naturally become arrested at metaphase of the second meiotic division (metaphase II, MetII). This review will concentrate

on how this arrest is achieved and in particular how the sperm breaks this arrest through a Ca^{2+} signal.

Exiting metaphase

In both mitosis and in MetII, sister chromatids need to be separated. The following is a summary of the main events of mitosis but further detail can be found in other reviews (Morgan 1999, Pines & Rieder 2001, Peters 2002, Uhlmann 2004). Sister chromatid segregation needs to be equal to maintain the correct ploidy. When sister chromatids congress on a metaphase spindle they become fully aligned (bi-oriented). Occupancy of all the kinetochores with microtubules and the associated pulling tension at kinetochores acts as a signal for anaphase to commence. At this time key cell cycle proteins that have been maintaining metaphase are degraded. Exit from metaphase is achieved by the Anaphase-Promoting Complex/Cyclosome (APC/C) a multi-subunit E3 ligase complex that has the ability to tag its substrates with the small molecular weight protein, ubiquitin (Morgan 1999, Zachariae & Nasmyth 1999, Peters 2002). Polyubiquitination of APC/C substrates leads to their immediate proteolysis through the 26S proteasome (Ciechanover 2005).

Figure 1 Schematic of the two meiotic divisions. (A) In the first meiotic division homologue pairs that have recombined (hence a mix of maternal: pink and paternal: blue, chromatin) are pulled apart by microtubules which form attachment to centrosomal chromatin by kinetochores (green). Before anaphase, tension develops as exchanged homologues remain attached by cohesin molecules (red) that are distal to the kinetochore. At anaphase onset activation of separase is triggered and the Rec8 component of cohesin proteolytically cleaved. Loss of cohesion in the arms allows the microtubule pulling forces to initiate poleward movement of homologues. During meiosis I, cohesin at the centromeres is protected from degradation (red shaded oval). (B) In the second meiotic division sister chromatids are kept attached by centromeric cohesin and at fertilization a sperm-derived Ca^{2+} signal activates separase to allow sisters to separate. For this to occur protection of centromeric cohesin is lost after meiosis I.

Two key APC/C substrates are cyclin B1 and securin. Cyclin B1 is the regulatory component of the essential mitotic/meiotic kinase Maturation (M-Phase) Promoting Factor (MPF). This heterodimer contains a CDK1/cdc2 catalytic subunit and a regulatory cyclin B1 subunit (Doree & Hunt 2002). MPF activity is necessary in order for a cell to enter mitosis and its destruction is needed for mitotic progression past metaphase. At metaphase, polyubiquitination of cyclin B1 by APC/C rapidly decreases MPF activity and in so doing allows anaphase. Sister chromatids are held together by cohesin complexes that are believed to form in a ring-like structure around the sister chromatids, physically preventing their separation (Haering *et al.* 2002). At anaphase the Scc1 subunit of cohesin is cleaved by separase, an enzyme that is normally held in an inactive state by securin during prometaphase (Uhlmann 2004). APC/C activation at metaphase results in degradation of securin and release of separase.

The first meiotic division

In mammalian eggs a rise in MPF activity induces exit from the prophase I arrest (Ledan *et al.* 2001). Its activity

continues to rise during the first meiotic division, declines with first polar body extrusion and abruptly returns as the egg re-arrests at MetII (Fig. 2). The decrease in MPF activity as oocytes exit meiosis I is necessary to allow for segregation of homologous chromosomes, in an analogous manner to that in mitosis and again is driven by cyclin B1 degradation (Ledan *et al.* 2001, Herbert *et al.* 2003, Hyslop *et al.* 2004). Interestingly, in contrast to mitosis, in frog oocytes during meiosis I, APC/C activity and so cyclin B1 degradation, appears dispensable such that completion of the first meiotic division can occur without cyclin B1 degradation and also in the absence of the essential APC/C activator at metaphase cdc20 (Peter *et al.* 2001, Taieb *et al.* 2001). However non-degradable cyclin B1 blocks mouse oocytes at MetI, suggesting that APC/C mediated cyclin B1 degradation is essential for meiosis I completion (Herbert *et al.* 2003, Hyslop *et al.* 2004). Obviously what is happening during meiosis I in the frog warrants further investigation, but it seems that in mammals at least, APC/C-mediated degradation of cyclin B1 is needed for homologous chromosome segregation. Degradation of securin is also required for separation of homologues, in that a non-degradable

Figure 2 MPF and MAP kinase (MAPK) activity during the two meiotic divisions. MPF activity (blue) rises abruptly at germinal vesicle breakdown (GVB). Its activity declines briefly at the time of first polar body extrusion (Pb1) and during the short interkinesis period MPF activity is re-established at a high level. MPF activity remains high during MetII arrest until a sperm-derived Ca^{2+} signal (sp.) induces degradation of cyclin B1 and so loss in MPF activity by the time of second polar body extrusion (Pb2). MAPK activity (red) rises with a lag as compared to MPF, but its activity remains stable and does not fall until just before pronucleus formation (PN). The x-axis is in hours after injection of superovulating hormone.

securin construct that is no longer an APC/C substrate prevents first polar body extrusion and homologue disjunction (Herbert *et al.* 2003). Therefore during first meiosis both cyclin B1 and securin need to be efficiently degraded and a failure to degrade fully either protein leads to an arrest at MetI.

Many errors in meiosis result from a mis-segregation during the first meiotic division, the clinical consequences of which include conditions such as trisomy 21 (Hassold & Hunt 2001). It is therefore of great importance to find the factor which controls the timing of cyclin B1 and securin degradation in mammalian oocytes. It has long been assumed that the control mechanisms were different during meiosis I, a theory supported by the lack of an essential role for the APC/C during frog meiosis I. It is therefore somewhat disappointing that meiosis I in mammals appears to be more similar to mitosis, in requiring both the APC/C and the spindle assembly checkpoint (SAC) to correctly segregate homologues. In mitosis the SAC prevents premature activation of the APC/C by recruiting components of the Mad and Bub families and Mps1 to kinetochores that are either unattached or lack tension (Shah & Cleveland 2000, Yu 2002, Taylor *et al.* 2004). Some studies have suggested that the SAC is not very active during meiosis I (LeMaire-Adkins *et al.* 1997), a hypothesis that would have underpinned the concept that meiosis I generates far more errors in segregation than is normally observed in mitosis. By preventing premature APC/C activation the SAC allows full congression and alignment of sister chromatids on the metaphase spindle before allowing anaphase. In this way daughter cells receive one of each pair of sister chromatids. If this pathway was absent or not as functional in meiosis I then one could see how chromosomes would, stochastically, incorrectly segregate, leading to events such as trisomy.

However several more recent publications on mouse oocytes during meiosis I have demonstrated that the SAC is active at this time (Brunet *et al.* 2003, Terret *et al.* 2003, Homer *et al.* 2005). Inhibition or impairment of the SAC, most directly by knockdown approaches of SAC family members, has shown that activation of the SAC normally occurs during the period of prometaphase I and that an inactive SAC leads to gross errors in segregation during meiosis I. Obviously it cannot be disputed that there are high error rates in the process of segregation at meiosis I (Hassold & Hunt 2001), but the underlying cause is not yet known, although it is interesting to note that the transcripts for Mad2 and Bub1, both critical components of the SAC, are lower in oocytes from older women (Steuerwald *et al.* 2001).

If protein synthesis is blocked during mouse meiosis I, oocytes fail to maintain condensed chromatin and so form a nucleus (Clarke & Masui 1983). This is because of the requirement for high MPF levels to maintain condensed chromatin and the fact that most cyclin B1 gets degraded at the end of the meiosis I. Indeed introduction of excess cyclin B1 into oocytes prolongs, or even prevents, anaphase I (Ledan *et al.* 2001, Hyslop *et al.* 2004). It is still interesting that during meiosis most cyclin B1 is degraded, MPF levels drop to low levels and in some instances it is at the same level as in a GV stage oocyte (Hashimoto & Kishimoto 1988, Choi *et al.* 1991, Fulka *et al.* 1992, Polanski *et al.* 1998) however there is no chromatin decondensation at this time. In frog it appears that MPF levels do not drop as much as in mouse and indeed that residual MPF levels are required during the transition from meiosis I to II (sometimes this period is referred to as interkinesis). In mouse oocytes during interkinesis it is thought that MAP kinase, whose activity is high in meiosis I oocytes (Fig. 2), substitutes for MPF activity. MAP kinase recognises a similar

peptide sequence to MPF and so may share substrates. However when the gene of *c-mos*, a MAP kinase kinase, is knocked out, oocytes lack completely MAP kinase activity but only partially enter an interphase stage immediately following first polar body extrusion (Verlhac *et al.* 1996). Therefore it remains possible that other factors in the meiosis I oocyte ensure continued chromatin condensation between meiosis I and MetII arrest.

Establishing a Metaphase II arrest

Having extruded the first polar body, the oocyte must now re-establish a high MPF level and remain arrested at MetII until fertilized. Oocytes have been ascribed an activity, Cytostatic Factor (CSF) that confers an ability to arrest chromatids at MetII (Masui & Markert 1971). The ability of the frog egg cytoplasm to confer arrest on a recipient cell suggests CSF is a diffusible factor, although its exact composition is still debated. Mouse eggs also possess CSF: fusion experiments between mouse eggs and embryos generate fused cells in which the embryonic chromatin becomes arrested at metaphase (Kubiak *et al.* 1993). There is much evidence to suggest that the product of the gene *c-mos* is responsible for CSF activity in both frog and mouse eggs (Tunquist & Maller 2003). The most compelling evidence is that *mos* injection into frog and mouse embryos induces a metaphase arrest and that removal of *c-mos* from frog or mouse eggs prevents a MetII arrest. In mouse either the removal of *mos* by generation of a $-/-$ knockout or by using antisense approaches, generates eggs that fail to arrest at MetII (O'Keefe *et al.* 1989, Colledge *et al.* 1994, Hashimoto *et al.* 1994, Stein *et al.* 2003).

How does *mos* mediate MetII arrest? The ability of *mos* to act as a MAP kinase kinase kinase implicates the MAP kinase pathway in CSF-induced egg arrest. Indeed MAP kinase becomes activated during mouse egg maturation and peaks at MetII (Verlhac *et al.* 1994). Furthermore pharmacological MAP kinase inhibitors can prevent a MetII arrest and will induce egg activation when added to eggs (Phillips *et al.* 2002).

The ultimate control point in the maintenance of metaphase arrest is in the prevention or the slowing down of cyclin B1 and securin degradation. Since both these proteins are degraded at anaphase-onset by polyubiquitination through *cdc20*-bound APC/C (APC^{cdc20}) and the 26S proteasome there are at least 3 points of possible CSF induced metaphase arrest (Fig. 3, labelled A, B and C). The first (A) is the most upstream control point and would control the level of cyclin B1 and securin synthesis. Therefore MetII arrest is maintained by increased synthesis of cyclin B1/securin. The fact that cyclin B1 synthesis is known to be up regulated during meiotic maturation (Tay *et al.* 2000) and that parthenogenetic activation follows incubation of eggs with protein synthesis inhibitors (Siracusa *et al.* 1978), suggests that this mechanism is not without merit. However recent experiments in which cyclin B1 and securin have been tagged with green fluorescent protein (GFP) all show

that their destruction is speeded up at fertilization (Nixon *et al.* 2002, Madgwick *et al.* 2004). Therefore if increased synthesis plays a part in establishing MetII arrest, it is not important in the process by which exit from MetII is achieved. Most research on MetII arrest is focussed on how the level of degradation is controlled and assumes that changes in synthesis are unimportant in MetII arrest. The second control point is at the level of the APC/C, either directly by negative regulation of the APC/C or indirectly by affecting the ability of *cdc20* to switch on the APC/C. This is by far the most preferred mechanism for reasons discussed later. The third control point (C) is at the level of the 26S proteasome and here it is reduced proteasome activity that prevents degradation of polyubiquitinated cyclin B1/securin.

Ca^{2+} : the egg activator

CSF-induced MetII arrest of eggs is broken by a sperm-derived Ca^{2+} signal. Given the redundancy often observed in signalling pathways it is surprising that the process of egg activation is dependent only on a rise in the egg cytoplasmic Ca^{2+} concentration. Therefore intracellular Ca^{2+} chelation blocks all the processes associated with egg activation, while Ca^{2+} mimetics induce full egg activation (Jones 1998).

The mechanism by which sperm trigger Ca^{2+} spiking has been a matter of controversy and has historically been polarised into two opposing schools of thought. The first school suggests that sperm provide a soluble factor, which upon sperm-egg fusion is released into the egg. The second school suggests that the sperm binds an egg plasma membrane receptor. Excellent recent reviews exist on this controversy and they all argue in favour of a soluble factor, which is a reflection of how the field currently stands (Runft *et al.* 2002, Rogers *et al.* 2004, Malcuit *et al.* 2005). A major breakthrough was the finding that mammalian sperm contain a sperm-specific phospholipase C isoform (PLC ζ), which is present at a sufficient concentration to induce Ca^{2+} spiking in the egg (Saunders *et al.* 2002). Recent studies have begun to address how this particular isoform of PLC is so suited to causing Ca^{2+} spiking in eggs (Kouchi *et al.* 2005, Kurokawa *et al.* 2005, Nomikos *et al.* 2005), whereas other PLC isoforms, such as PLC γ , are only able to induce spiking at very high concentrations (Mehlmann *et al.* 2001). It will be interesting to determine how evolutionary conserved is PLC ζ , given the long history of studying Ca^{2+} release in eggs at fertilization in many species (Stricker 1999). One study has shown that chicken sperm contains PLC ζ (Coward *et al.* 2005) and interestingly shares a bidirectional promotor with the testis-specific gene CAPZA3, as is the case with mammalian PLC ζ . CAPZA3 is intronless and therefore likely to be a retogene. It is tempting to speculate that the evolutionary emergence of PLC ζ occurred with a retroposon genome insertion of CAPZA3. Although

Figure 3 Control points for Ca^{2+} action during cell cycle resumption at fertilization. In MetII arrested eggs there is measurable amount of APC/C activity that continues to degrade cyclin B1 and securin via polyubiquitination and so proteolysis through the 26S proteasome. In theory there are at least 3 possible control points for Ca^{2+} to stimulate this process: (A) by blocking the synthesis of cyclin B1 and securin synthesis, (B) by stimulating the APC/C^{cdc20} and (C) by stimulating the 26S proteasome. (B) is by far the most likely control point.

present in sperm at sufficient concentration it is still to be formally proven that PLC ζ is physiologically involved in Ca^{2+} release at fertilization. The fertility of a PLC ζ knock-out mouse is still to be reported in the literature. A transgenic RNA interference approach (Knott *et al.* 2005), only partially reduced PLC ζ levels in a population of sperm because the extent of knockdown varied from sperm to sperm. However, interestingly, no transgenic offspring were observed using transgenic founder males following mating suggesting that sperm with very reduced PLC ζ levels, due to effective RNAi, failed to produce live pups. Thus far, this is the best evidence to show that PLC ζ is physiologically important at fertilization.

In frog eggs the sperm generates a 10 minute long Ca^{2+} wave that is propagated across the egg cytoplasm, whereas in contrast, the 3–5 minute first Ca^{2+} rise in mammalian eggs (that also traverses as a wave) is then followed by a series of Ca^{2+} spikes that last 4–6 hours (Jones 1998, Jones *et al.* 1998). The long-lasting Ca^{2+} spiking in mammalian eggs is thought to ensure that eggs fully activate. Single Ca^{2+} spikes have the ability to induce partial egg activation: eggs undergo second polar body

extrusion but the chromatin re-arrests on a monopolar third spindle (Kubiak 1989). This is probably due to continued CSF activity re-establishing a metaphase arrest in the absence of Ca^{2+} spiking. Partial activation (the metaphase III state) occurs with greatest frequency in freshly ovulated eggs that are stimulated to activate by a short Ca^{2+} signal, i.e. single pulse. Interestingly such short Ca^{2+} signals can fully activate eggs that are aged above the normal window of fertilization occurring *in vivo*. This suggests the following possibilities: firstly there is a decline in CSF activity as eggs age, secondly aged eggs have a reduced ability to reactivate CSF, and thirdly cyclin B1 levels/MPF activity decline with egg age. Any of these possibilities would likely lead to a single Ca^{2+} spike being an effective parthenogenetic agent to induce full egg activation.

Ca^{2+} in the resumption of meiosis

Two important downstream targets of Ca^{2+} action at fertilization are cyclin B1 and securin. In promoting the

polyubiquitination and so proteolysis, of cyclin B1, MPF levels are lowered and cell cycle progression out of metaphase is made possible. When cyclin B1 has its Destruction (D)-box motif – responsible for APC/C-mediated degradation – removed, then eggs fail to degrade cyclin B1 at fertilization (Madgwick *et al.* 2004). As a consequence cell cycle resumption is prevented, there is no second polar body extrusion or pronucleus formation. Similar APC/C-mediated degradation of securin allows the release of separase, that then acts on the cohesin complex which holds sister chromatids together at metaphase. In mitosis it is Scc1 that is cleaved by separase, allowing the separation of sister chromatids (Uhlmann 2004). In meiosis, Scc1 is largely replaced by Rec8 (Xu *et al.* 2005) and it remains an interesting question as to how Rec8 is degraded from sister arms in meiosis I to allow segregation of homologous chromosomes, but is protected at centromeres until sisters separate at anaphase II (Marston & Amon 2004). Little is known about this process in mammalian eggs, although it has been shown that securin degradation is essential for normal anaphase I and II (Herbert *et al.* 2003, Madgwick *et al.* 2004). Failure to degrade securin during exit from meiosis II, leads to an inability of eggs to fully segregate their sister chromatids. Chromatin is stretched between the second polar body and the egg (Madgwick *et al.* 2004), presumably due to persistence in securin activity and consequently a lack of separase-mediated cohesin cleavage, a phenotype that has been observed in other cells using a non-degradable securin (Zur & Brandeis 2001, Hagting *et al.* 2002).

It is not known if cyclin B1 and securin are the only two essential substrates to ensure meiotic exit in mammalian eggs. One may speculate that CSF itself should be a target of the sperm-triggered Ca^{2+} spikes. Indeed in mouse eggs CSF activity declines quickly when eggs are activated, with similar dynamics to the loss in MPF activity (Ciemerych & Kubiak 1999). However there is no a priori reason for CSF to decline at the same time as cyclin B1 and securin. This is because Ca^{2+} may by-pass the CSF-mediated egg arrest to induce degradation of these APC/C substrates. In the frog this indeed does seem to be the case, since CSF activity does not decline until after MPF levels have declined (Watanabe *et al.* 1991). In mammalian eggs the chromatin has the capacity to remain condensed after second polar body extrusion and re-establish a metaphase arrest, rather than fully decondense and form a pronucleus, if Ca^{2+} spiking is terminated prematurely (Kubiak 1989, Collas *et al.* 1993, Collas *et al.* 1995). This suggests that in the mammalian egg although CSF may initially dissipate, it also has the capacity to be re-activated when a Ca^{2+} signal is absent.

APC/C: the control point for Ca^{2+} action

It is possible to visualise and quantitate cyclin B1 and securin degradation in eggs during fertilization by

coupling them to GFP and introducing the cRNA chimera constructs. When this is done a very large increase in degradation, about 6-fold, is observed around 10 minutes after the first sperm-induced Ca^{2+} spike (Nixon *et al.* 2002). Degradation continues until a minimum level is reached just before second polar body extrusion and it is not until around the time that pronuclei form (4–6 hours after the first Ca^{2+} spike), that cyclin B1 and securin levels start to rise again. Consistent with APC/C-mediated degradation, when the D-box of cyclin B1 is removed then cyclin destruction is not accelerated by Ca^{2+} (Madgwick *et al.* 2004).

How is the Ca^{2+} signal promoting degradation of these APC/C substrates? One possibility is that the 26S proteasome, responsible for the proteolysis of polyubiquitinated APC/C substrates, is in a quiescent state during MetII and that the Ca^{2+} signal augments its catalytic function. Although the activity of the 26S proteasome has been measured to increase during activation in eggs of some species (Kawahara *et al.* 1992, Kawahara & Yokosawa 1994, Aizawa *et al.* 1996), direct measurement of proteasome activity in mouse eggs failed to demonstrate any changes in proteasomal activity (Hyslop *et al.* 2004). The most likely control point is therefore at the level of the APC/C itself. It is probable that MetII arrest mediated by CSF is in some way down regulating – but not abolishing – APC/C activity and that Ca^{2+} restores full APC/C activity, thereby promoting anaphase by decreasing both cyclin B1 and securin protein levels.

Control of the APC/C by Ca^{2+} is an interesting signalling pathway, given the fact that Ca^{2+} is a ubiquitous ion, involved in many signalling events. Also given its roles in the cell cycle, and indeed even in post-mitotic cells (Konishi *et al.* 2004), the APC/C must be near-universally present in cells. It is therefore interesting to speculate if it is only at fertilization that there is an interaction between Ca^{2+} and the APC/C. Nature re-uses and modifies extant proteins/pathways for new cell functions, therefore it would seem possible that Ca^{2+} may control the APC/C in other processes. To support this hypothesis is the fact that Ca^{2+} spiking has been reported during mitosis in other cell types (Whitaker & Larman 2001). However, the presence of Ca^{2+} spikes is not essential for somatic mitosis (as it is in MetII), such that many cells readily undergo mitosis in the absence of detectable Ca^{2+} changes (Kao *et al.* 1990). Interestingly, this observation is also true of the first meiotic division, where both cyclin B1 and securin are degraded in an APC/C-dependent manner, at least in mouse, in the absence of any detectable Ca^{2+} changes (Hyslop *et al.* 2004, Marangos & Carroll 2004). Furthermore Ca^{2+} has no ability to speed up their destruction, as it does at MetII. The two key differences in these two cell cycle states would be: (a) the physical make up of the spindle, i.e. what is being segregated (homologous chromosomes versus sister chromatids) and (b), the establishment of a metaphase arrest at MetII but not during normal meiosis I. Interestingly what appears to establish Ca^{2+}

sensitivity is not the nature of the spindle and hence what is being segregated, but rather the fact that oocytes have arrested. This is deduced by the observation that in a subset of maturing oocytes that fail to complete the first meiotic division and in so doing arrest with attached homologs, addition of Ca^{2+} causes completion of the first meiotic division, as does Ca^{2+} for MetII (Hyslop *et al.* 2004).

Switching off the APC/C for MetII arrest using the spindle checkpoint pathway

How does *mos* switch off the APC/C for MetII arrest? The 90 KDa ribosomal protein S6 kinase (p90^{Rsk}) seems an important downstream target of the *mos*/MAP kinase pathway. The ability of the protein kinase p90^{Rsk} to be activated by MAP kinase is known in a number of biological systems and indeed it appears to form a heterodimer with MAP kinase (Bhatt & Ferrell 2000). In frog egg extracts, removal of this kinase prevents these extracts from arresting at metaphase and addition of a constitutively active kinase can induce a MetII arrest in eggs treated with the MAP kinase kinase inhibitor U0126 (Bhatt & Ferrell 1999, Gross *et al.* 2000). Also introduction of constitutively active p90^{Rsk} arrest blastocysts at metaphase, consistent with a CSF activity (Gross *et al.* 1999). *Rsk2*, one of the main four members of *Rsk* family, is the predominant *Rsk* found in frog eggs and appears to be the isoform responsible for MetII arrest (Bhatt & Ferrell 2000). Interestingly it was reported that removal of p90^{Rsk} did not induce cell cycle resumption in metaphase-arrested extracts (Bhatt & Ferrell 1999), suggesting that establishing MetII arrest and maintaining it are not necessarily the same process. However, in contrast to frog, a recent study in mouse, suggests that in mammalian eggs *Rsk*s have little role in establishing MetII arrest (Dumont *et al.* 2005). Thus constitutively active *Rsk1* and *Rsk2* were unable to re-establish a MetII arrest in *mos*^{-/-} eggs and furthermore eggs from a triple *Rsk* knockout (*Rsk*s 1, 2 and 3) all arrest at MetII. The one remaining *Rsk*, *Rsk4*, could not be detected in eggs. These data strongly argue that in mouse, *Rsk*s do not mediate the actions of *mos* and that *Rsk*s are not involved in establishing a MetII arrest.

In the frog, *Rsk* may work by activating Bub1, a member of the SAC. In frog eggs Bub1 is activated during meiosis and this activation is driven by p90^{Rsk} (Schwab *et al.* 2001). This Bub1 activation by *Rsk* may generate CSF activity and if so, is complicated by the fact that this event may recruit further SAC components, some of which may be essential for setting up the CSF arrest but not for maintaining it (Tunquist *et al.* 2003). Thus both Mad1 and Mad2, members of the SAC, are essential in setting up a *mos*-mediated metaphase arrest, but only Mad1 is needed for maintaining it once established (Tunquist *et al.* 2003). On the basis that there is clear evidence for SAC components in CSF-mediated arrest, it is interesting to

determine what components of the SAC are also needed to establish a MetII arrest in mammalian eggs. Surprisingly the one recent study that has examined the role of Bub1, Mad2 and BubR1, further SAC components, in mouse MetII arrest, provide no evidence that these SAC components establish or maintain MetII arrest (Tsurumi *et al.* 2004). Their experimental approach was to prevent Bub1, Mad2 and BubR1 action by injecting dominant negative versions of these constructs into prophase oocytes and then mature them to MetII. Interestingly all these constructs speeded up meiosis I, suggesting that the SAC plays a role in the timing of the first meiotic division, but that MetII is still established in these eggs. Thus for mouse it seems unlikely that MetII arrest is established or maintained by these SAC components. However one caveat is that these SAC dominant negative components may retain CSF activity, but not SAC function. Such a finding was observed for Mad2, in which a mutant incapable of forming oligomers lost its CSF function, but retained that of its SAC (Tunquist *et al.* 2003).

Switching off the APC/C for MetII arrest using Emi1 and Erp1

Emi1 is an attractive CSF candidate that acts at the level of the APC/C. Emi1 appears to have a role in preventing APC/C activity in G2 and early prophase of the somatic cell cycle (Reimann *et al.* 2001a, 2001b) by inhibiting the ability of *cdc20* to activate the APC/C. Removal of Emi1 protein from CSF-arrested frog extracts by immunodepletion failed to greatly affect *cdc20* levels (~80% *cdc20* remained in the extract) but did induce cyclin B degradation (Reimann & Jackson 2002). Overexpression of *cdc20* protein overcame CSF arrest in frog extracts consistent with a model in which CSF arrest is maintained by an Emi1 mediated APC/C inhibition, preventing *cdc20* from binding and activating the APC/C (Reimann & Jackson 2002).

There appears to be an important association between *Rsk2* and Emi1 that does not involve SAC components. A recent study has established that *Rsk2* can phosphorylate Emi1 and this phosphorylation is important in activating Emi1 (Paronetto *et al.* 2004). Phosphorylated Emi1 increases its binding to *cdc20* fourfold and it was argued that this may be the mechanism of *Rsk2* mediated MetII arrest. This study is important because it effectively unites two important CSF candidates that initially seem unrelated: *mos* and Emi1. From the study by Paronetto and co-workers, one could hypothesise that the mechanism of CSF arrest is *mos*-induced activation of Emi1, mediated by *Rsk2*. However given the fact that eggs from the *Rsk* triple knockout mouse all arrest normally at MetII this hypothesis seems now less likely (Dumont *et al.* 2005).

The role of Emi1 in MetII still remains to be fully established not only in mammalian eggs but also in frog.

In frog eggs Emi1 induced MetII arrest can be overcome by over expression of cdc20, a dose effect that is consistent with its mode of action (Reimann & Jackson 2002). However over expression of cdc20, or introduction of cdc20 mutants that have increased activity relative to wild-type into intact mouse eggs, has no effect on either the establishment or maintenance of MetII arrest (Tsurumi *et al.* 2004). Maybe mouse MetII arrest is different from that in frog. However it has also been questioned in frog if Emi1 is present in MetII arrested eggs (Ohsumi *et al.* 2004). Since the two main studies into the role of Emi1 in frog egg MetII arrest used different antibodies (Reimann & Jackson 2002, Ohsumi *et al.* 2004) it remains to be established if eggs possess more than one Emi isoform, One of which may be present at MetII (as in Reimann & Jackson 2002) and the other degraded at prophase (the normal timing of Emi1 destruction in the mitotic cell cycle, as in Ohsumi *et al.* 2004). Interesting, recently an Emi1 related protein (Erp1 or Emi2), was found to be present in frog eggs and appears to have many CSF properties through its ability to inhibit the APC/C (Schmidt *et al.* 2005). Importantly Erp1 degradation is Ca^{2+} -dependent, making Erp1 the most currently attractive candidate to mediate CSF arrest. Clearly all of the above suggests further studies are required to establish the relative role of the various candidates both in the establishment and the maintenance of MetII arrest.

Calmodulin-dependent protein kinase II and protein kinase C: downstream effectors of Ca^{2+} ?

In frog eggs calmodulin-dependent protein kinase II (CamKII) is the downstream effector of Ca^{2+} action at fertilization. A constitutively active CamKII induces cell cycle resumption and conversely a pseudosubstrate peptide of CamKII blocks escape from MetII arrest (Lorca *et al.* 1993). In mouse eggs a similar CamKII mediated pathway is likely to transduce the Ca^{2+} signal at fertilization since CamKII levels rise during fertilization and inhibitors block egg activation (Tatone *et al.* 2002, Markoulaki *et al.* 2004). However, interestingly the same observations have been made with respect to protein kinase C (PKC) (Colonna *et al.* 1997, Gallicano *et al.* 1997, Luria *et al.* 2000). Therefore this suggests that in mouse eggs there may be some redundant signalling downstream of Ca^{2+} . Critically however, the role of both CamKII and PKC in transducing cell cycle resumption during mouse fertilization is limited to pharmacological intervention. These pharmacological tools may have other actions that impinge on egg activation, for example KN-93, a well-used CamKII inhibitor appears to be able to inhibit Ca^{2+} release through the inositol trisphosphate (IP_3) receptor by a mechanism that is independent of its action on CamKII (Smyth *et al.* 2002). Therefore KN-93 inhibits cell cycle resumption in mouse, not by pharmacological inhibition of CamKII, but by blocking Ca^{2+} release at the level of the

IP_3 receptor (Smyth *et al.* 2002, Madgwick *et al.* 2005). One recent study has however shown that microinjection of constitutively active CamKII, but not PKC α , can induce cell cycle resumption into mouse eggs (Madgwick *et al.* 2005). This suggests that in both frog and mouse eggs CamKII is responsible for cell cycle resumption. Could other isoforms of PKC still be responsible for a redundant mechanism of cell cycle resumption? If other isoforms are involved, then these would have to be one of the conventional Ca^{2+} sensitive PKC isoforms (PKC β or γ). However this is unlikely given that PKC β is absent in some strains of mouse (Pauken & Capco 2000) and the PKC γ knockout mouse is normally fertile (Abeliovich *et al.* 1993). Although these observations argue against a role for PKC directly in the signal transduction pathway leading cell cycle resumption, PKC may have other roles at fertilization. Recent data point to PKC being involved in opening Ca^{2+} channels on the egg plasma membrane necessary for Ca^{2+} store refilling (Halet *et al.* 2004). During each Ca^{2+} spike there is an associated loss of Ca^{2+} due to efflux, a process that has to be compensated for by Ca^{2+} entry (McGuinness *et al.* 1996). Therefore PKC plays an essential role in maintaining Ca^{2+} spiking (Halet *et al.* 2004, Madgwick *et al.* 2005). Addition of PKC constructs or PKC activators to eggs was observed to speed up Ca^{2+} spiking due to increased Ca^{2+} entry, or with constitutively active PKC induce a sustained high intracellular Ca^{2+} due to failure of the egg to be able to close its plasma membrane Ca^{2+} channels (Halet *et al.* 2004, Madgwick *et al.* 2005). Conversely PKC inhibitors reduce the frequency of spiking (Halet *et al.* 2004).

The Ca^{2+} -stimulated CamKII activity is likely to activate the APC/C at fertilization, thereby stimulating degradation of cyclin B1, securin and possibly other substrates important in MetII arrest. It seems that many studies have focussed logically on cdc20 being the likely control point for cell cycle arrest, be it by activation of the SAC or through Emi1/Erp1. The observation that over expression of cdc20 active constructs fail to overcome a MetII arrest in mouse is therefore an enlightening one (Tsurumi *et al.* 2004) and would appear to suggest that activity of other APC/C components may be important at MetII. The fact that the APC/C is a multi-subunit complex makes the task of identifying the key components daunting, made even more so by the very complex pattern of phosphorylation observed for APC/C during mitosis (Kraft *et al.* 2003).

Future perspectives

Despite extensive research, much is still to be understood at the molecular level of how eggs, particularly mammalian eggs, achieve an arrest at metaphase II and how the Ca^{2+} signal at fertilization breaks this arrest. Work is needed to understand how CamKII interacts with the APC/C. It is an immensely interesting subject for study. The ability of eggs to arrest with a fully aligned, bioriented

spindle, challenges some of the cell cycle dogma which would dictate that if this were a mitotic division then the APC/C would be switched on at this time and anaphase would commence. Furthermore the need for a signalling cue (Ca^{2+}) to break this arrest brings together two areas of research that do not overlap extensively. Once the molecular identity of some of these key players is resolved, it will be interesting to determine if they are involved in any analogous cell cycle processes reliant on Ca^{2+} signals that possibly lie outside of embryology as these may be important in developmental biology or cell cycle control in adult cells. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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