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Second meiotic arrest and exit in frogs and mice

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Mature vertebrate oocytes typically undergo programmed arrest at the second meiotic cell cycle until they are signalled to initiate embryonic development at fertilization. Here, we describe the underlying molecular mechanisms of this second meiotic arrest and release in *Xenopus*, and compare and contrast them with their counterparts in mice.

Keywords: metaphase II; Emi2; cytostatic factor; vertebrate meiosis; fertilization

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Introduction

Mature oocytes of vertebrates such as *Xenopus laevis* (African clawed frog) and *Mus musculus* (mouse) are arrested at the second metaphase (mII) of the meiotic cell cycle. Second meiotic progression is restrained by cytostatic factor (CSF), the presence of which was originally inferred from the ability of mII oocyte extracts from *Rana pipiens* (the northern leopard frog) to induce cleavage arrest when injected into dividing blastomeres (Masui & Markert, 1971). CSF activity sustains mII arrest in fertilizable oocytes to prevent parthenogenesis. Here, we compare the maintenance of, and exit from, mII arrest in the oocytes of frogs and mice (Table 1); a composite schematic summarizing the molecular interactions implicated in these processes is shown in Fig 1.

An overview of metaphase II arrest

Metaphase correlates with the kinase activity of maturation promoting factor (MPF), which is a heterodimer of cyclin B (Cycb) and the cyclin-dependent kinase, Cdc2 (also known as Cdk1; Masui & Markert, 1971; Gautier *et al,* 1989, 1990). MPF is indirectly stabilized by active CSF and its substrates include the linker histone H1 (Gautier *et al,* 1989). Although MPF is active in both mitotic and meiotic cell cycles, stabilization by CSF is unique to mII; CSF compensates for the negative feedback by MPF that typically predominates in mitotic cells, in which the activation of MPF leads to its own inactivation (Félix *et al,* 1990; Kubiak *et al,* 1993).

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MPF activity is inhibited in prophase I—that is, in immature oocytes—through the phosphorylation of Cdc2 residues Thr14 and Tyr15. This reflects the net activities of the MPF-activating phosphatase, xCdc25c (Cdc25b in the mouse), and the MPF-inhibitory kinases xWee1a (Tyr15 phosphorylation) and xMyt1 (Thr14 and Tyr15 phosphorylation), which are supplanted by Wee1b in the mouse (Leise & Mueller, 2002; Han *et al,* 2005). In *Xenopus*, germinal vesicle breakdown is prevented by the action of cAMP-dependent protein kinase A (PKA), which phosphorylates Ser287 of xCdc25 until meiosis is resumed (Duckworth *et al,* 2002). However, there is no firm evidence in either the mouse or *Xenopus* that the pathways involving PKA, Cdc25, Wee1 or Myt mediate mII maintenance or exit. In *Xenopus*, CSF-induced metaphase arrest occurs only when the spindles are correctly attached to the kinetochores—the spindle assembly checkpoint (SAC); the SAC-mediating protein, xMad1, is required to maintain mII (Tunquist *et al,* 2003). By contrast, the SAC is not required to establish or to maintain mII arrest in the mouse (Tsurumi *et al,* 2004).

Exit from mII occurs when the destruction box motif of Cycb directs ubiquitination by an E3 ubiquitin ligase called the anaphase promoting complex (APC), a complex of at least 12 protein subunits (Peters, 2006). Ubiquitination targets Cycb for 26S proteasomal hydrolysis to eliminate MPF (Glotzer *et al,* 1991; Peters, 2006). The main effect of CSF is therefore to stabilize MPF by abrogating APC activity towards Cycb. In prevailing models, APC inhibition involves the oocyte-restricted endogenous meiotic inhibitor 2 (Emi2), the activity of which is essential for CSF arrest. The events after gamete fusion that precede syngamy are referred to as oocyte activation (Runft *et al,* 2002).

Emi2 and Mos in meiosis II

Xenopus endogenous meiotic inhibitor 2 (xEmi2, also known as Emi1-related protein 1, xErp1) is a 651-residue protein that mediates mII establishment and/or maintenance (Fig 1; Schmidt *et al,* 2005; Rauh *et al,* 2005). During CSF arrest, xEmi2 apparently negates APC activity by binding to the core of the APC, one component of which is xCdc27 (Wu *et al,* 2007a). This interaction reflects the balance of xEmi2 phosphorylation by xCycb–xCdc2 (MPF; Wu *et al,* 2007a,b), and the antagonistic removal of these phosphates (Nishiyama *et al,* 2007a; Inoue *et al,* 2007; Wu *et al,* 2007a).

The search for the CSF-promoting contribution to this balance effectively began with classical experiments in which *xMos* complementary RNA (cRNA) injected into the blastomeres of *Xenopus* two-cell embryos induced cleavage arrest (Sagata *et al,* 1989).

Table 1 | Some disparities between the oocytes and early embryos of *Xenopus* and *Mus*

Property	*Xenopus*	*Mus*	References
Mature oocyte volume	1 μl	270 pl	–
Spindle assembly checkpoint requirement for mII arrest	Yes	No	Tunquist *et al*, 2003; Tsurumi *et al*, 2004
Intact spindle requirement for maturation promoting factor destruction	No	Yes	Kubiak *et al*, 1993; Clute & Masui, 1995
Activation by pricking in Ca^{2+}-containing medium	Yes	No	Wolf, 1974
Protein synthesis inhibition induces mII exit	No	Yes	Zhang & Masui, 1992; Siracusa *et al*, 1978
Cytostatic factor activity demonstrated by injecting cleaving blastomeres with (≥12% of their volume) mII ooplasm	Yes	No	Shibuya & Masui, 1988; Shoji *et al*, 2006
Oocytes support nuclear transfer cloning of adults from adult-derived somatic cells	No	Yes	Gurdon, 1960; Wakayama *et al*, 1998
Full chromatin decondensation requires activation	Yes	No	Yoshida *et al*, 2007
Activation accompanied by an increase in intracellular pH	Yes	No	Webb & Nuccitelli, 1981; Phillips & Baltz, 1996
$[Ca^{2+}]_i$ oscillations induced by fertilization	No	Yes	Runft *et al*, 2002
Zygotic gene activation	4,000-cell stage	Late 1-cell stage	Aoki *et al*, 1997; Newport & Kirschner, 1982

xMos immunodepletion reduced the ability of mature oocyte extracts to induce CSF arrest (Sagata *et al,* 1989), and depletion of xMos by morpholino injection of intact *Xenopus* oocytes prevented mitogen-activated protein kinase (Mapk) activation and induced parthenogenesis (Dupré *et al,* 2002). Therefore, meiotic arrest in *Xenopus* involves an xMos–xMek–xMapk (xErk1/2) pathway. Pharmacological inhibition of xMek with U0126 induces mII exit (Wu *et al,* 2007a) in a manner that can be bypassed by the expression of constitutively active xRsk, a target of xMapk signalling (Gross *et al,* 2000). Indeed, xRsk phosphorylates xEmi2 primarily at Ser 335 and Thr 336, but also at Ser 342 and Ser 344 (Fig 2), with phosphorylation at Ser 335 and Thr 336 facilitating the binding of xEmi2 to protein phosphatase 2A (xPP2A; Wu *et al,* 2007a). In turn, xPP2A binding promotes dephosphorylation of xEmi2 residues Ser 213, Thr 239, Thr 252, Thr 267, Thr 545 and Thr 551. Dephosphorylation of Thr 545/Thr 551 enhances the binding of the xEmi2 carboxy-terminal zinc-binding region to the APC core, thereby inhibiting APC (Wu *et al,* 2007a). Dephosphorylation of the Ser 213–Thr 267 cluster, which is conserved or conservatively substituted in the mouse alignment (Fig 2), stabilizes xEmi2 (Wu *et al,* 2007b). Phosphorylation of xEmi2 Ser 213–Thr 267 and Thr 545/Thr 551 clusters, which tends to negate CSF activity, is mediated by MPF through a balancing negative feedback loop (Wu *et al,* 2007a,b).

xMos is a crucial Mapkkk that acts upstream from xRsk (Inoue *et al,* 2007; Nishiyama *et al,* 2007a), and exogenous xMos fails to induce metaphase arrest in interphase extracts from which xRsk2 has been immunodepleted (Bhatt & Ferrell, 1999). Therefore, xPP2A activity towards xEmi2 is stimulated by xMos through xRsk, to promote mII arrest. The xMos–xMapk–xRsk cascade permanently disappears after mII exit, whereas xEmi2 is destroyed during *Xenopus* mII exit and then reappears in pre-blastula cell cycles but without its CSF activity; the xMos–xMapk–xRsk pathway might therefore be required for the CSF activity of xEmi2 (Inoue *et al,* 2007). However, CSF extracts that are depleted of xRsk2 (in which it is approximately 16-fold more abundant than xRsk1) remain arrested in mII for at least 25 min (Bhatt & Ferrell, 1999), which is consistent with a role for xRsk-independent events to sustain MPF activity.

In the mouse, homozygously targeted *Mos*$^{-/-}$ oocytes fail to activate the Mapk pathway and pause at mII for 2–4 h, with initially normal MPF activity (Colledge *et al,* 1994; Choi *et al,* 1996; Verlhac *et al,* 1996). Oocytes that lack Mos contain anomalously long, interphase-like microtubules during the mI–mII and mII–mIII transitions (Verlhac *et al,* 1996). Coordination of the cell cycle and microtubule dynamics could involve the microtubule-stabilizing Mapk targets, Miss and Doc1r, although neither contributes directly to CSF activity (Lefebvre *et al,* 2002; Terret *et al,* 2003). However, a deficiency of Mos perturbs meiosis I, and subsequent phenotypes might reflect this.

In the mouse, constitutively active Rsk fails to stabilize mII arrest in *Mos*$^{-/-}$ oocytes, and oocytes homozygous for deletions in the three expressed *Rsk* genes (*Rsk1*, *Rsk2*, *Rsk3* triple-null mice) present a stable CSF arrest with classical barrel-shaped spindles (Dumont *et al,* 2005). Thus, Rsk-independent signalling pathways might stabilize mII in both the mouse and *Xenopus*.

Maturing and mII mouse oocytes also contain Emi2 (Shoji *et al,* 2006), which exhibits 44% amino-acid identity with xEmi2 over its 541-residue alignment (Fig 2). Emi2 is also expressed at high levels in the mouse testis, suggesting an as-yet-undetermined role in male meiosis (Shoji *et al,* 2006).

Emi2 functions by interfering with the APC activator, Cdc20. A C-terminal fragment of *Xenopus* xEmi2 inhibits xCdc20-dependent ubiquitination of a Cycb fragment *in vitro* (Schmidt *et al,* 2005). Cdc20 depletion in intact mouse oocytes prevents mII exit induced by *Emi2*-targeted RNA interference, and Emi2 and Cdc20 have been shown to interact *in vitro* (Shoji *et al,* 2006). However, *Xenopus* xEmi2 interacts only weakly with xCdc20, if at all (Schmidt *et al,* 2005; Wu *et al,* 2007a), raising the possibility that mouse oocyte Cdc20–Emi2 interactions are also weak. Cdc20 removal completely blocks mII exit induced by sperm or the parthenogenetic

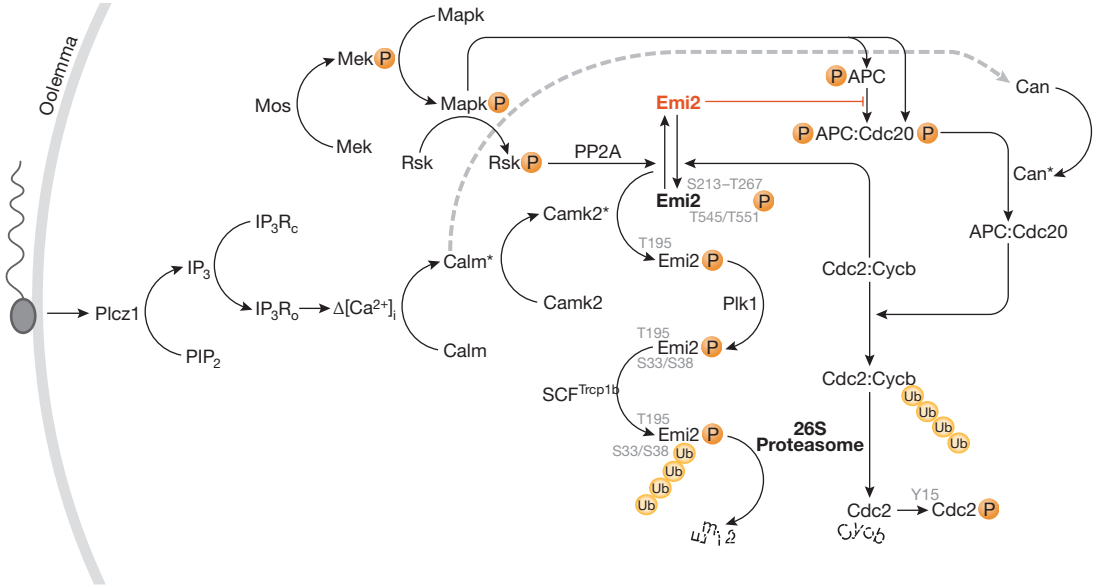

Fig 1 | Composite schematic of endogenous meiotic inhibitor 2 regulation in vertebrates. Details of selected interactions are described in the text. APC, anaphase promoting complex; Calm, calmodulin; Camk2, calmodulin-dependent kinase 2; Can, calcineurin; Cdc2/20, cell division cycle 2/20 homologues, respectively; Cdc2:Cycb, maturation promoting factor; Cycb, cyclin B; Emi2, endogenous meiotic inhibitor 2; IP_3, inositol 1,4,5-trisphosphate; IP_3R_c, closed IP_3 receptor; IP_3R_o, open IP_3 receptor; Mapk, mitogen-activated protein kinase; Mek, mitogen-activated protein kinase kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; Plcz1, phospholipase C-zeta; Plk1, polo-like kinase 1; PP2A, protein phosphatase 2A; Rsk, ribosomal s6 kinase; SCF, Skp1– Cullin– F-box-containing E3 ubiquitin ligase complex; Trcp1b, F-box protein β-transducin repeat containing protein 1b; Ub, ubiquitin; P, phosphate; *, active form; $\Delta[Ca^{2+}]_i$, increase in the intracellular free calcium ion concentration.

agent $SrCl_2$ *in situ* (Shoji *et al,* 2006), indicating that in the mouse, either all pathways regulating mII exit involve Cdc20, or that others are secondary to those that do.

Exit from mII: the inducing sperm signal

Sperm–oocyte fusion in *Xenopus* induces an increase in intracellular 'free' calcium ($[Ca^{2+}]_i$) that propagates across the oocyte before returning after 5 min to its resting level (Runft *et al,* 1999, 2002); there are no $[Ca^{2+}]_i$ oscillations. Mouse gamete fusion stimulates a marked $[Ca^{2+}]_i$ increase from the point of sperm–oocyte contact within 3 min (Lawrence *et al,* 1997). The $[Ca^{2+}]_i$ then returns to its resting level (~100 nM), but is followed by a series of oscillations lasting several hours in which $[Ca^{2+}]_i$ spikes to approximately 1 μM for about 1 min every 5–15 min (Runft *et al,* 2002).

The murine activity that induces oocyte $[Ca^{2+}]_i$ oscillations includes the phospholipase C (Plc) isoform, Plc-ζ (Plcz1; Saunders *et al,* 2002). Injection of *Plcz1* cRNA into mouse oocytes induces $[Ca^{2+}]_i$ oscillations and pronucleus formation (Saunders *et al,* 2002). Sperm protein profiling showed that Plcz1 correlates with a physiological oocyte activating factor (Fujimoto *et al,* 2004). Plcz1 orthologues are conserved among mammals but are unusual among Plcs because they lack plekstrin or Src homology (PH or SH) domains; proceeding from its amino- to C-termini, Plcz1 comprises four EF-hand domains, X and Y domains and a C-terminal C2 domain (Saunders *et al,* 2002). No *Xenopus Plcz1* counterpart has been confirmed and although the hypothetical protein NP_001090146.1 exhibits 42% identity along a 628-residue alignment with mouse Plcz1 (NP_473407; 647 residues), its expression pattern and activity have not been reported. Orthologues of Plc-β and Plc-γ isoforms are also present in *Xenopus* oocytes but are dispensable for activation (Runft *et al,* 1999).

Calcium signalling is relayed through xCamk2 in *Xenopus*

How is intracellular calcium signalling detected to induce mII exit? *Xenopus* egg extracts supplemented with a calmodulin-binding peptide prevent exogenous calcium from triggering xCycb degradation (Lorca *et al,* 1991). This implicit calmodulin-dependent regulation involves calmodulin kinase II (xCamk2), as challenging *Xenopus* eggs or their extracts with constitutively active rat Camk2a (the α-isoform; residues 1–290, lacking regulatory and subunit association domains, referred to here as Camk2aΔct) inactivates CSF (Hanson & Schulman, 1992; Lorca *et al,* 1993).

Native Camk2 activity in newly fertilized mouse eggs shadows $[Ca^{2+}]_i$ oscillations (Markoulaki *et al,* 2004) and injection of mouse mII oocytes with cRNA encoding Camk2aΔct results in meiotic progression without these oscillations (Knott *et al,* 2006). However, the Camk2 antagonist, myristoylated autocamtide 2-related inhibitory peptide, myrAIP, does not block early $[Ca^{2+}]_i$ oscillations or polyspermy, and incompletely prevents meiotic resumption after sperm–oocyte fusion (Markoulaki *et al,* 2004; Gardner *et al,* 2007).

Xenopus xCamk2 signals mII exit through xEmi2

In *Xenopus* oocyte extracts, xCamk2 phosphorylates xEmi2 at Thr 195 of its canonical Camk2 phosphorylation motif, RXST, in a manner facilitated by the preceding serine residue, Ser 194;

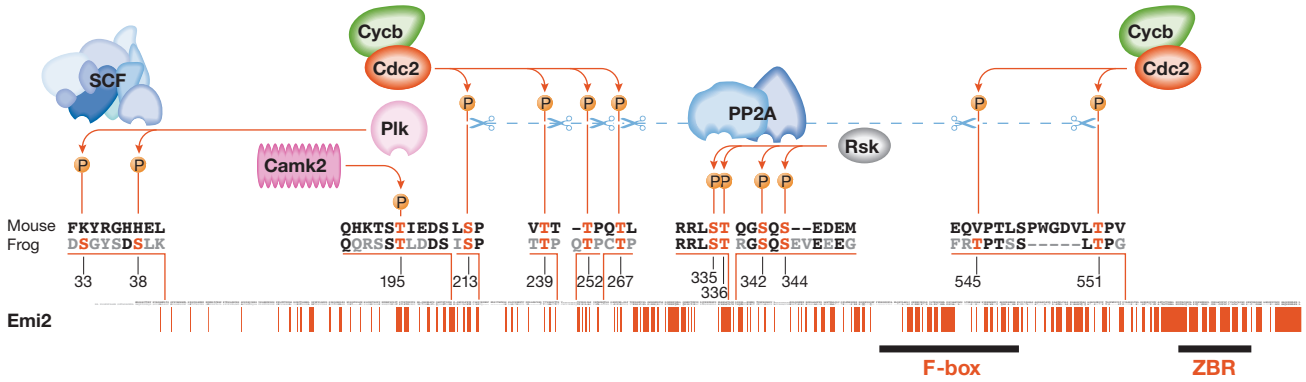

Fig 2 | Pairwise alignment of mouse (*Mus*, top) and frog (*Xenopus*, bottom) endogenous meiotic inhibitor 2 predicted amino-acid sequences, showing identities as red bars beneath. The alignment was based on one produced by BLAST. Key portions of the alignment (described in the text) are expanded, showing residues identical to the mouse sequence in black, non-identical ones in grey and those subject to phosphorylation in red, with their corresponding kinases and/or phosphatases. Information directing mII establishment and/or maintenance apparently resides in the carboxy-terminal 70%, with the amino-terminal 30% containing elements that direct mII exit. The F-box and the zinc-binding region (ZBR) are as delineated by Schmidt *et al* (2005). Abbreviations are as for Fig 1.

xEmi2^{T195A} is resistant to degradation in Ca^{2+}-supplemented CSF extracts (Rauh *et al,* 2005). A peptide corresponding to the Camk2 regulatory domain (Camk2$^{218–309}$) reduces Ca^{2+}-induced xEmi2 degradation, which supports a direct interaction between xEmi2 and xCamk2 (Rauh *et al,* 2005; Liu & Maller, 2005).

xEmi2 phosphorylated at Thr 195 is an improved substrate for the polo-like kinase, xPlk1 (also referred to as Plx1), and neither S194A nor T195A mutants interact with xPlk1 (Rauh *et al,* 2005). Immunodepletion of xPlk1 from CSF extracts prevents meiotic progression on addition of Ca^{2+} (Descombes & Nigg, 1998) and a marked (10- to 15-, but not twofold) excess of xPlk1 induces meiotic progression in the absence of Ca^{2+} (Liu & Maller, 2005). xEmi2 regulation involves the polo-box domain (PBD) of xPlk1, because a xPlk1 dominant-negative PBD stabilizes xEmi2 and inhibits CSF release in *Xenopus* extracts supplemented with Ca^{2+} (Schmidt *et al*, 2005).

The binding of xPlk1 to xEmi2 primed by Thr 195 phosphorylation facilitates the secondary phosphorylation of xEmi2 by xPlk1 at Ser 33 and Ser 38 within the phosphodegron motif DSGX_3S (Schmidt *et al,* 2005; Rauh *et al,* 2005). After these secondary DSGX_3S phosphorylations have occurred, xEmi2 is rapidly targeted by the Skp1–Cullin–F-box^{Trcpb}-containing E3 ubiquitin ligase complex, resulting in its 26S proteasomal hydrolysis (Schmidt *et al,* 2005).

Depletion of Emi2 from intact mouse mII oocytes causes meiotic release, and Emi2 degradation during oocyte activation precedes Cycb destruction (Shoji *et al,* 2006; Madgwick *et al,* 2006). Mouse Emi2 lacks the canonical RXX$^{T}/_{S}$ motif corresponding to the Camk2 phosphorylation target (which includes Thr 195) in its alignment with xEmi2 (Rauh *et al,* 2005). However, the arginine is conservatively substituted (by Lys 173 in mouse); the resultant KXX$^{T}/_{S}$ motif is phosphorylated by Camk2 in the smooth muscle contractility regulator, caldesmon (Hanson & Schulman, 1992). Although xEmi2 is phosphorylated by xPlk1 in its Ser 33/Ser 38 phosphodegron, the corresponding region is poorly conserved in the mouse and other mammals (Schmidt *et al,* 2005), and there is no canonical substitute nearby (Fig 2).

Calcineurin and mII exit in *Xenopus*

In addition to the involvement of xCamk2, the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin (xPpp3c; referred to here as Can) has recently been implicated in *Xenopus* mII exit (Mochida & Hunt, 2007; Nishiyama *et al,* 2007b). Can comprises a catalytic A subunit, Cana, tightly bound to a regulatory B subunit, Canb, which is bound to Ca^{2+}/calmodulin in active Can (Klee *et al,* 1998). When Ca^{2+} is added to CSF extracts, the APC core subunit xCdc27 (also known as xAPC3) becomes rapidly and transiently dephosphorylated in an xCamk2-independent manner that is inhibited by the indirect Can inhibitors, cyclosporin A (CsA) or FK506 (Mochida & Hunt, 2007). CsA also inhibits xCycb destruction, and depletion of Can reduces xCdc2 kinase inactivation in response to Ca^{2+} (Mochida & Hunt, 2007; Nishiyama *et al,* 2007b). Can apparently dephosphorylates xCdc27 and xCdc20; dephosphorylation of xCdc20 is rapid and might 'unlock' it to contribute to APC activation (Mochida & Hunt, 2007).

Little is known about Can or its relationship to Camk2-induced mII progression in mouse oocytes. They might be part of the same pathway, as Can is a substrate of Camk2 (Hanson & Schulman, 1992), or Can-mediated reactions immediately triggered by a rise in $[Ca^{2+}]_i$ might be independent of Camk2, as seems to be the case in frogs (Nishiyama *et al,* 2007b). In the mouse, Camk2aΔct only weakly establishes the block to polyspermy and does not fully induce cortical granule exocytosis (Gardner *et al,* 2007); these are early events of activation in which Can might have a significant role. Can is activated in response to small changes in $[Ca^{2+}]_i$, which might explain why *Xenopus* oocytes are readily activated by the influx of external calcium ions that often accompanies injection or pricking (Wolf, 1974); mouse oocytes are not activated in this manner.

A low-resolution model of mII homeostasis

We propose a simple model of meiosis II in which Mos activity senses and modulates correct spindle behaviour to potentiate the stable establishment of mII arrest by Emi2 (Fig 3).

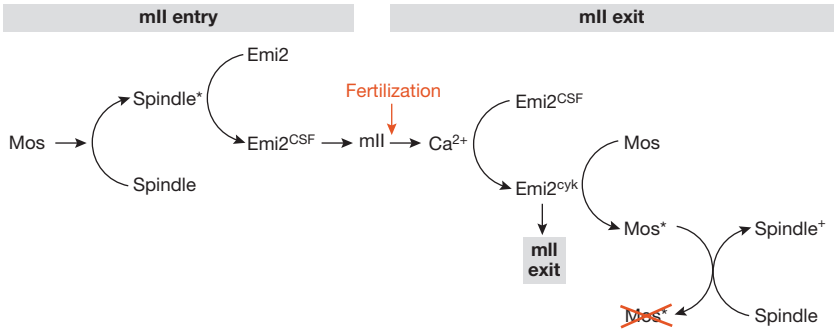

Fig 3 | Low-resolution model of second metaphase homeostasis. The model links spindle modulation by Mos to the establishment and maintenance of, and exit from, Emi2-mediated mII arrest. The establishment of Emi2 as cytostatic factor ($Emi2^{CSF}$) is coordinated by mII spindle formation (spindle*) and Mos activity. Following fertilization, $Emi2^{CSF}$ is replaced by $Emi2^{cyk}$ activity which modulates Mos function (Mos*) to ensure that the spindle is formatted for productive cytokinesis ($spindle^{+}$).

In the model, Mos-mediated microtubule adjustments during the transition from meiosis I to II result in a Mapk-utilizing, Rsk-independent signal to Emi2 to establish mII (Bodart *et al,* 2005). The features of Emi2, Mos and microtubules are linked in several ways. Emi2 localizes to spindles in both *Xenopus* and mouse maturing oocytes (Madgwick *et al,* 2006; Ohe *et al,* 2007). Mouse Mapk regulates microtubule organization and asymmetrical division (Yu *et al,* 2007), and the absence of Mos perturbs Mapk signalling, inducing microtubule elongation between meiotic divisions (Verlhac *et al,* 1996). A further indication that Mos and spindle dynamics are linked comes from the world of microRNA (miRNA); in mouse oocytes, a functional miRNA network is required both for spindle maintenance and the regulation of *Mos* mRNA (Tang *et al,* 2007).

Mouse Cycb degradation and mII exit also require an intact spindle; microtubule disruption prevents meiotic progression (Winston *et al,* 1995). This suggests that signalling through Camk2, Can or other activating pathways is insufficient to guarantee mII exit and is subordinate to at least one independently regulated mechanism involving microtubules, possibly through Plk1, which is dynamically associated with mII spindles (Tong *et al,* 2002). Mos might also modulate Emi2 activity during mII exit, as $Mos^{-/-}$ mII oocytes often collapse to mIII following exogenous activation (Araki *et al,* 1996). Mos and Emi2 could be associated with macromolecular assemblies that proximate elements of the Mapk pathway, Cycb/Cdc2, Plk1, tubulin and motors required for spindle positioning with respect to cortical actin. It remains to be seen whether Mos or Emi2 are constituents of a macromolecular assembly in either *Xenopus* or the mouse.

Our appreciation of CSF largely derives from work using *Xenopus* oocyte extracts, which is a powerful system to analyse biochemical interactions and signalling pathways. Research on intact mouse oocytes suggests that regulation of the spindle—a pivotal macromolecular assembly—might be essential for mII maintenance and exit. In the future, it will be important to characterize the interactions between microtubules, Emi2 and the Mos–Mapk pathway. In particular, we should learn whether the spindle directs local Mos–Mapk activation and if so, the extent to which this modulates Emi2 activity and other processes that are significant for mII homeostasis.

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