Cytostatic factor: an activity that puts the cell cycle on hold

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Summary

Fertilization is the fundamental process in which two gametes – sperm and oocyte – fuse to generate a zygote that will form a new multicellular organism. In most vertebrates, oocytes await fertilization while arrested at metaphase of meiosis II. This resting state can be stable for many hours and depends on a cytoplasmic activity termed cytostatic factor (CSF). Recently, members of the novel Emi/Erp family of proteins have been put forward as important components of CSF. These proteins inhibit the anaphase-promoting complex/cyclosome (APC/C), which acts at the very core of the cell cycle regulatory machinery. Initially, *Xenopus* early mitotic inhibitor 1 (Emi1) was proposed to be a component of CSF, but newer work suggests that a structural relative, Emi-related protein 1 (Erp1/Emi2), is essential for maintenance of CSF arrest in *Xenopus*. Most importantly, studies on Erp1/Emi2 regulation have led to a detailed molecular understanding of the Ca^{2+}-mediated release from CSF arrest that occurs upon fertilization.

Key words: Cytostatic factor, Cell-cycle regulation, APC/C, Erp1/Emi2, Meiosis, Oocyte fertilization

Introduction

Vertebrate oogenesis leads to the production of immature oocytes, which arrest (in some species for years!) at the first meiotic prophase. Upon hormonal stimulation these oocytes then undergo 'maturation' and enter the meiotic cell cycle (Ferrell, 1999) (see Fig. 1). In frogs, mice, humans and many other vertebrates meiosis halts again at metaphase of meiosis II, yielding a fertilizable (so-called mature) egg. On fertilization, the egg quickly completes the second meiotic division to generate a haploid pronucleus that can then fuse with the male pronucleus to form a diploid zygote. In their classic study, more than three decades ago, Masui and Markert found that the cytoplasm of mature eggs contains two different biochemical activities that cause distinct effects when injected into cells (Masui and Markert, 1971). The first can induce maturation when injected into immature oocytes (i.e. trigger entry into the meiotic cell cycle in the absence of hormone) and was therefore referred to as maturation-promoting factor (MPF), now known to be a complex of cyclin-dependent kinase 1 (Cdk1) and cyclin B (Nurse, 2002). The second causes cell cycle arrest when injected into a mitotically dividing embryo, leading to its designation as cytostatic factor (CSF) (see Fig. 1). This arrest is characterized by the presence of a metaphase spindle and high Cdk1 activity in the injected cell, just like the physiological CSF arrest that occurs in meiosis II. Unlike MPF, CSF has long resisted detailed biochemical characterization or purification, and defining its molecular composition has been difficult (Shibuya and Masui, 1988). However, the extensive research conducted on CSF has led to the definition of criteria that molecules involved in CSF activity must fulfil: (1) they should appear during oocyte maturation; (2) they should be present and functional at metaphase of meiosis II; and (3) they should be inactivated upon fertilization or parthenogenetic activation of the egg.

In 1989 Sagata and co-workers proposed that the proto-oncogene Mos, a germ-cell-specific protein kinase uniquely induced at the beginning of oocyte maturation, is responsible for CSF arrest in *Xenopus* eggs (Sagata et al., 1989) (for a review, see Sagata, 1997). Subsequently, a pathway comprising downstream mediators of Mos-induced CSF arrest was characterized and shown to include a mitogen-activated protein kinase (MAPK) module containing the MEK and Erk1/2 kinases, the 90 kDa ribosomal subunit S6 kinase ($p90^{RSK}$) and components of the spindle-assembly checkpoint, particularly the vertebrate orthologues of the yeast mitotic arrest deficient (Mad) and budding uninhibited by benzimidazole (Bub) proteins (Bhatt and Ferrell, 1999; Gross et al., 1999; Haccard et al., 1993; Tunquist et al., 2003; Tunquist et al., 2002). In addition, an independent pathway involving Cdk2–Cyclin-E was characterized through an antisense oligonucleotide approach (Gabrielli et al., 1993), although these results have subsequently been challenged by the observation that injection of the Cdk2 inhibitor $p21^{CIP}$ does not interfere with CSF arrest (Furuno et al., 1997).

All CSF pathways are thought to inhibit ultimately a ubiquitin ligase called the anaphase-promoting complex/cyclosome (APC/C) (Lorca et al., 1998; Tunquist and Maller, 2003; Vorlaufer and Peters, 1998). The APC/C is a large assembly of proteins that associates with one of at least two activators, Cdc20 or Cdh1, to direct polyubiquitylation of securin, cyclins and other cell cycle regulators for subsequent degradation by the proteasome. Cdc20 recognizes substrates early in mitosis, whereas Cdh1 targets substrates later in mitosis and during the following G1 phase (for review, see Peters, 2002).

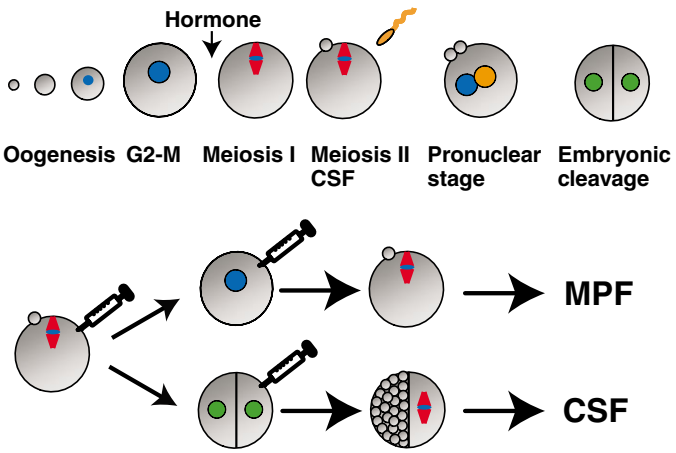

Fig. 1. MPF and CSF are key activities in vertebrate oocytes. The upper panel summarizes stages important for oocyte development and the onset of embryonic development. Oogenesis leads to the production of an immature oocyte arrested in prophase of meiosis I. After resumption of meiosis in response to hormonal stimulation, the oocyte progresses until the second arrest point in meiosis II (CSF arrest). Fertilization releases the oocyte from this arrest and triggers exit from meiosis II. The lower panel shows a simplified scheme illustrating the cytoplasmic injection experiments that led to the identification of MPF and CSF in frog oocytes. Small amounts of cytoplasm taken from mature oocytes were injected into immature oocytes or one blastomere of two-cell embryos. After injection immature oocytes resume meiosis whereas injected blastomeres arrest in metaphase. These observations led Masui and Markert to postulate that cytoplasm of mature oocytes contains two distinct biochemical activities, MPF and CSF, that regulate the oocyte maturation process (Masui and Markert, 1971).

Extensive studies by Maller and co-workers (Tunquist and Maller, 2003) indicate that the Mos/MAPK/p90^{RSK} pathway inhibits APC/C by activating a subset of components (notably Bub1) (Schwab et al., 2001) of the spindle assembly checkpoint (SAC), which normally prevents the onset of anaphase following spindle damage in mitotically dividing cells (Musacchio and Hardwick, 2002; Yu, 2002). However, the observation that Mad2 is dispensable for maintenance of CSF indicates that Mos mediates CSF arrest in a manner distinct from the spindle checkpoint (Waters et al., 1998; Tunquist et al., 2003).

These pathways implicated in CSF activity have been expertly reviewed elsewhere (Maller et al., 2002; Tunquist and Maller, 2003) and, therefore, we only briefly summarize them here. Instead, we focus the following discussion on more recent advances in our understanding of CSF activity. These stem primarily from the discovery of a novel class of inhibitors of the APC/C, the Emi/Erp family (Reimann et al., 2001; Schmidt et al., 2005), and culminate in the unraveling of the mechanisms that lead to Emi-related protein 1 (Erp1, also known as Emi2) inactivation in response to fertilization (Liu and Maller 2005; Rauh et al., 2005; Hansen et al., 2006). We emphasize findings in *Xenopus*, since it is in this model system that these proteins and regulatory mechanisms have been most thoroughly characterized.

The Emi/Erp family

The vertebrate Emi/Erp family so far comprises only two known members, Emi1 and Erp1/Emi2, both of which can directly inhibit the APC/C. Emi1 homologues have been identified and functionally characterized in *Xenopus,* human and mouse (Guardavaccaro et al., 2003; Hsu et al., 2002; Paronetto et al., 2004; Reimann et al., 2001). These species also possess Erp1/Emi2 homologues, but only the *Xenopus* protein (XErp1) has been investigated in some detail (Schmidt et al., 2005; Tung et al., 2005). *Drosophila* regulator of cyclin A 1 (Rca1) is a potential invertebrate relative of Emi1 and Erp1. However, although Rca1 is implicated in the negative regulation of APC/C activity (Dong et al., 1997; Grosskortenhaus and Sprenger, 2002), possible differences in the mechanisms underlying oocyte meiotic arrest in *Drosophila* (Ivanovska et al., 2004) make it difficult to assess the precise role of Rca1 in this process and further studies will be required to determine its relationship with Emi1 and Erp1.

The founding member of the family is Emi1, which was first identified in an unbiased screen for human F-box-containing proteins and thus named FBXO5 (Cenciarelli et al., 1999). F-Box proteins are known to be part of Skp1/cullin/F-box protein (SCF) ubiquitin ligase complexes which, like the APC/C, serve to target cell cycle regulators for destruction. A large number of different F-box proteins associate with the SCF complex via the Skp1 subunit (which is itself an F-box protein) and act as specificity factors to mediate the ubiquitylation of target proteins (Vodermaier, 2004). *Xenopus* Erp1 (XErp1), also called FBXO43 or Emi2, was identified in a yeast two-hybrid screen aimed at finding substrates of *Xenopus* polo-like kinase 1 (Plx1) (Schmidt et al., 2005). Phylogenetic analysis of mammalian F-box-containing proteins shows that there is a close relationship between human Erp1/Emi2 and Emi1 (Jin et al., 2004).

In addition to their C-terminal F-box domains, both Emi1 and Erp1/Emi2 harbor a zinc-binding region (ZBR) (see Fig. 2), which is essential for both proteins to inhibit the ubiquitin-ligase activity of the APC/C (Reimann et al., 2001; Schmidt et al., 2005). Note, however, that even though both Erp1 and Emi1 seem to be bona fide F-box proteins – i.e. both bind to the F-box-binding protein Skp1 (Reimann et al., 2001) (P. I. Duncan and E.A.N., unpublished data), which implies that they are components of SCF complexes – there is as yet no functional evidence for such activity in vivo. In fact, none of the functions so far been described for Emi1 or XErp1/Emi2 requires the F-box.

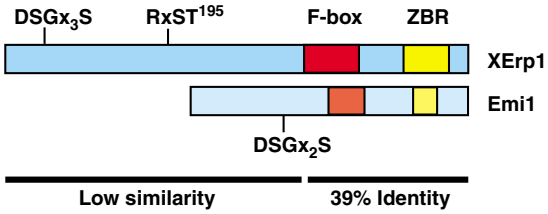

Fig. 2. XErp1 and *Xenopus* Emi1. Domains and positions of amino acid sequences are depicted approximately to scale. XErp1/Emi2 and *Xenopus* Emi1 share 39% identity in their C-terminal domains. The C-terminus of both proteins contains the F-box followed by a zinc-binding region (ZBR). Note that the XErp1 N-terminal extension is not shared by Emi1.

Emi1: a mitotic APC/C inhibitor

Upon entry into mitosis, cells must activate Cdk1 to induce processes such as nuclear envelope breakdown, chromosome condensation and bipolar spindle assembly. However, rising Cdk1 activity also promotes the activation of the APC/C by inducing the recruitment of the activator Cdc20 (Kramer et al., 2000), which might potentially lead to premature destruction of cyclins. Thus, cells may have evolved mechanisms to prevent cyclin degradation prior to the activation of the SAC. From this perspective, the discovery of Emi1 provided an attractive explanation for how the APC/C could be kept inactive at the beginning of mitosis. Emi1 could be synthesized in S phase and bind to and thereby inhibit the APC/C activator Cdc20 so that mitotic cyclins can accumulate for activation of MPF (Reimann et al., 2001). Later in mitosis, Emi1 could then be destroyed in a ubiquitin-dependent manner, allowing full APC/C activation. Further studies indicated an even earlier role for human Emi1, suggesting that its E2F-dependent accumulation at the G1-S boundary promotes S-phase entry in human cells by preventing APC/C^{Cdh1}-dependent degradation of cyclin A (Hsu et al., 2002). These studies on Emi1 function and regulation thus seem to justify the designation ‘early mitotic inhibitor’. By contrast, no mitotic functions have yet been described for Erp1/Emi2.

Emi1 and CSF activity

How does Emi1 relate to CSF activity in vertebrate oocytes? Given its ability to inhibit the APC/C, Emi1 appeared to be a prime candidate for a key component of CSF. Indeed, Jackson and co-workers have shown that injection of the protein into one cell of a two-cell embryo causes the arrest of the injected cell with high Cdk1 activity and, furthermore, reported that *Xenopus* Emi1 is both necessary and sufficient for CSF arrest in *Xenopus* egg extracts (Reimann et al., 2001; Reimann and Jackson, 2002). Using antibodies thought to be specific for Emi1, they showed that depletion of the protein from CSF-arrested *Xenopus* egg extracts caused premature APC/C activation in the absence of a Ca^{2+} signal. Moreover, excess Emi1 prevented the release of extracts from CSF arrest, which can be experimentally induced by the addition of $CaCl_2$ (a procedure that mimics fertilization of the intact egg).

Early doubts about whether Emi1 is a CSF component have been raised by two observations, however. First, the addition of Emi1 to mitotic *Xenopus* egg extract prevents exit from mitosis by stabilizing both cyclin A and B (Reimann et al., 2001), which is different from Mos-imposed CSF arrest, in which only cyclin B but not cyclin A is stabilized (Tunquist and Maller, 2003). Second, the observed timing of Emi1 degradation in somatic cells seemed to be incompatible with Emi1 being a CSF component. Jackson and co-workers initially reported that *Xenopus* Emi1 is not degraded after fertilization but persists throughout the first mitotic division (Reimann et al., 2001). However, subsequent studies revealed that the ubiquitin ligase $SCF^{\beta\text{-}TRCP}$ targets Emi1 for degradation in mitotic prophase (Hansen et al., 2004; Margottin-Goguet et al., 2003; Moshe et al., 2004). These apparent failures to satisfy the CSF criteria (first, Emi1 stabilizes both cyclin A and B in mitosis; and, second, it is degraded during prophase and so should not be present at metaphase) could in principle be explained if one assumes that Emi1 behaves differently in meiosis and mitosis. However, the function of Emi1 as a CSF component was further called into question when Kishimoto and colleagues reported that endogenous Emi1 is undetectable in CSF-arrested *Xenopus* oocytes (Ohsumi et al., 2004). Furthermore, these authors showed that exogenous Emi1 protein is unstable in maturing oocytes and CSF extracts, that non-degradable versions of Emi1 interfere with meiotic progression and that Emi1-mediated arrest is independent of the classic MAPK-mediated CSF pathway (Ohsumi et al., 2004). Although some of these findings do not necessarily rule out a contribution of Emi1 to CSF activity, the absence of Emi1 from the egg would obviously exclude such a role.

Erp1/Emi2: does the second born explain it all?

Resolution of this problem began when XErp1/Emi2 was characterized as a Plx1-regulated APC/C inhibitor essential for CSF activity (Schmidt et al., 2005). At about the same time, some antibodies originally used to study Emi1 were found also to recognize XErp1/Emi2; this suggested that the premature CSF release observed upon addition of such ‘Emi1-specific antibodies’ could actually have been caused by inhibition of XErp1/Emi2 function (Tung et al., 2005). Schmidt and colleagues showed that the depletion of XErp1/Emi2 from CSF extract with an antibody directed against its N-terminus leads to Ca^{2+}-independent, premature release from CSF arrest, but that the arrest is maintained upon simultaneous addition of a C-terminal fragment of XErp1/Emi2 that is able to directly inhibit the ubiquitylation activity of APC/C (Schmidt et al., 2005). Importantly, the antibody used in these depletion experiments is specific for XErp1/Emi2, because it recognizes the N-terminus of XErp1/Emi2, which is not shared by Emi1 (see Fig. 2). These results thus demonstrated that XErp1/Emi2 is essential to maintain CSF arrest. Experiments also showed that endogenous XErp1/Emi2 is rapidly destroyed after addition of Ca^{2+} to CSF extracts, which is consistent with XErp1/Emi2 being a component of CSF (Schmidt et al., 2005; Tung et al., 2005). This Ca^{2+}-triggered degradation of XErp1/Emi2 depends on Plx1, which phosphorylates a motif known to serve as a phospho-dependent recognition signal for the ubiquitin ligase $SCF^{\beta\text{-}TRCP}$. In previous studies, the addition of catalytically inactive Plx1 to CSF extracts had been shown to prevent CSF inactivation through a dominant-negative mode of action (Descombes and Nigg, 1998; Liu et al., 2004). Remarkably, the inactivation of XErp1/Emi2 is sufficient to overcome the block induced by catalytically inactive Plx1, which indicates that XErp1/Emi2 is the relevant substrate of Plx1 in the regulation of exit from meiosis II (Schmidt et al., 2005).

Jackson and co-workers have independently confirmed that XErp1/Emi2 is sufficient to prevent CSF release and that it is a target of Plx1 (Tung et al., 2005). In contrast to Ohsumi and colleagues, however, they maintain that Emi1 protein is present in CSF extract together with XErp1/Emi2. Using four different affinity-purified antibodies raised against a full-length Emi1 fusion protein they report detection and immunoprecipitation of a protein that migrates with its predicted molecular weight of ~44 kDa. However, owing to the lack of an Emi1-specific antibody they could not study the functional significance of this observation.

What is the function of Ca^{2+} in CSF release?

It has been known for more than a decade that the Ca^{2+} signal induced by sperm entry activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in the egg (Lorca et al., 1993). However, the relevant downstream target(s) that liberates the APC/C from CSF-imposed arrest upon activation of CaMKII has remained obscure. Likewise, a requirement for Plx1 during CSF release was established several years ago (Descombes and Nigg, 1998), but the relationship between Plx1 and the Ca^{2+} signal had remained mysterious. Recently, two laboratories have provided insight into the molecular mechanism of CSF release by demonstrating that CaMKII triggers the degradation of XErp1/Emi2 in *Xenopus* egg extract through the induction of Plx1 recruitment onto XErp1/Emi2 (Liu and Maller, 2005; Rauh et al., 2005). In particular, Rauh and colleagues showed that CaMKII phosphorylates XErp1/Emi2 at threonine 195 (T195), which then leads to enhanced binding of Plx1 to XErp1/Emi2. In turn, Plx1 phosphorylates a $DSGx_3S$ sequence in the N-terminus of XErp1/Emi2 and thereby creates a recognition motif for the ubiquitin ligase $SCF^{\beta\text{-}TRCP}$, causing XErp1/Emi2 destruction. Mutation of T195,S194 (the serine residue crucial for Plx1 binding) or the $DSGx_3S$ motif renders XErp1/Emi2 resistant to Ca^{2+}-induced degradation. These data identify CaMKII as a priming kinase for Plx1 and clarify how the Ca^{2+} signal controls the timing of XErp1/Emi2 degradation. They also explain why XErp1/Emi2 remains stable in CSF extracts in which both Plx1 and $SCF^{\beta\text{-}TRCP}$ are active. Thus, a temporal signal (a rise in Ca^{2+} levels) has been combined with spatial regulation (phosphorylation-dependent targeting leading to degradation) to link fertilization to the completion of meiosis II (see Fig. 3).

Rauh and co-workers also found that, in contrast to XErp1/Emi2, the degradation of Emi1 in anaphase extract does not depend on CaMKII activity (Rauh et al., 2005). This would be consistent with a previous report claiming that Emi1 remains stable after fertilization until the first mitotic division (Reimann et al., 2001). So, if Emi1 were indeed present in CSF extract and to contribute to CSF (see Ohsumi et al., 2004), then its activity would have to be downregulated upon fertilization by a mechanism other than degradation. So far, no such mechanism has emerged. Thus, the available evidence identifies Emi1 as an APC/C inhibitor that has an important role in the mitotic cell cycle, but there is presently no conclusive evidence for a role of *Xenopus* Emi1 in CSF arrest.

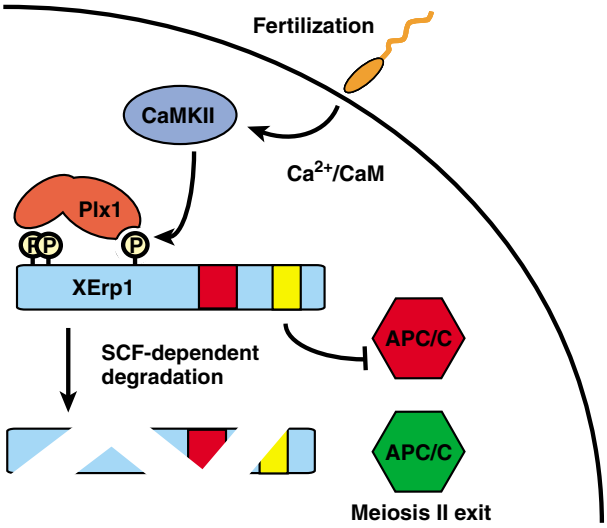

Fig. 3. Model for meiosis II exit after fertilization-induced destruction of XErp1. During CSF arrest, the APC/C is kept inactive by XErp1. Fertilization leads to the production of intracellular Ca^{2+} transients that activate CaMKII. CaMKII phosphorylation on Thr195 directs Plx1 to its substrate XErp1. Plx1 phosphorylation then triggers $SCF^{\beta\text{-}TRCP}$-dependent degradation of XErp1 by the ubiquitin/proteasome system. As a result, the APC/C is liberated from its repression and triggers meiosis II exit.

CSF activity and oocyte maturation

Upon exposure to progesterone, immature frog eggs proceed through the first meiotic division until they arrest at metaphase of meiosis II. The fact that maturing oocytes do not arrest during meiosis I indicates that CSF is not functional at that time, and each potential CSF candidate must satisfy this criterion. Unfortunately, data concerning the function or regulation of Emi1 or XErp1/Emi2 during the first meiotic division are scarce. There is evidence that both proteins are present in immature oocytes just before entry into meiosis (Reimann et al., 2001; Schmidt et al., 2005). Although the abundance and phosphorylation state of Emi1 seem to remain unchanged during oocyte maturation (Reimann et al., 2001), XErp1/Emi2 undergoes post-translational modifications at the meiosis I to meiosis II transition (Schmidt et al., 2005). Immature oocytes injected with anti-Emi1 antibodies fail to enter meiosis I upon progesterone treatment, which indicates that Emi1 might have a function promoting oocyte maturation (Tung and Jackson, 2005). However, once again, the interpretation of these experiments might be complicated by the issue of reagent specificity – i.e. the antibody used might crossreact with XErp1/Emi2. Consequently, a major question that remains unanswered is how and when exactly Emi1 and XErp1/Emi2 become active during oocyte maturation.

A unified theory of CSF arrest?

How do the classical MAPK-mediated CSF pathway and the novel CSF components described here interact? There is still some debate as to whether the MAPK pathway is required only to establish CSF arrest or also to maintain it (Bhatt and Ferrell, 1999; Furuno et al., 1997; Reimann and Jackson, 2002; Yamamoto et al., 2005). Emi1 and XErp1/Emi2 function do not appear to be strictly dependent on full MAPK activity (Reimann and Jackson, 2002; Schmidt et al., 2005). However, the experiments addressing this point used exogenous proteins and addition of MAPK inhibitors to *Xenopus* egg extract. It is conceivable, therefore, that residual MAPK activity persisting under these conditions might have been sufficient to ensure APC/C inhibition in the presence of large amounts of Emi1 or XErp1/Emi2 proteins. Thus, it would be premature to exclude the possibility that the MAPK kinase pathway plays a role in the activation of Emi/Erp family members. Indeed, Paronetto and co-workers reported that Emi1 and $p90^{RSK}$, a downstream target of the Mos/MAPK pathway, functionally interact in mouse oocytes (Paronetto et al., 2004). They further proposed that $p90^{RSK}$-mediated phosphorylation of Emi1 enhances its binding to the APC/C

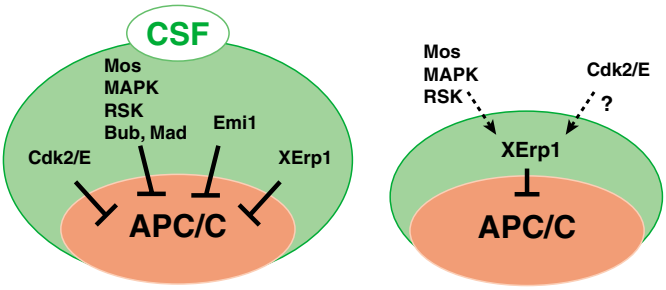

Fig. 4. Models of CSF arrest. The establishment and maintenance of CSF may involve many different, seemingly parallel pathways (left panel). However, we speculate that future investigation and reinvestigation of all proposed components could lead to a more straight-forward model in which all pathways converge onto the APC/C inhibitor Erp1 (right panel).

activator Cdc20 and thereby ensures APC/C inhibition during CSF arrest. However, these results have been called into question by a recent analysis of knockout mice lacking all three $p90^{RSK}$ isoforms (Dumont et al., 2005). Oocytes of these mice arrest normally at metaphase of meiosis II and show normal meiotic spindle morphology; this indicates that, at least in mice, $p90^{RSK}$ proteins are not required for CSF arrest. Furthermore, injection of mRNA encoding constitutively active $p90^{RSK}$ does not restore CSF arrest in Mos-knockout oocytes. Intriguingly, however, injection of $p90^{RSK}$ into two-cell-stage mouse embryos also fails to induce metaphase arrest, although analogous injections into *Xenopus* embryos stop division. This indicates that there may be significant differences in the way different species regulate CSF arrest. In the future, it will clearly be important to clarify the connection between the MAPK pathway and Emi/Erp family proteins in different species.

Conclusion

Research on CSF arrest has made a big step forward with the identification of the Emi/Erp family of proteins. Erp1/Emi2 has emerged as a major player directly ensuring APC/C inhibition, and consequent cell cycle arrest, at metaphase of meiosis II. The regulated destruction of Erp1/Emi2 after fertilization is likely to be the primary event that triggers release from CSF arrest, completion of meiosis and the subsequent onset of embryonic development. However, important unresolved questions remain. In particular, the establishment of CSF arrest presumably involves some sort of activation of Erp1/Emi2 and it would be premature to exclude the possibility that this activation involves the Mos-MAPK pathway. Intimately linked to this issue is the question of whether multiple pathways independently lead to CSF arrest (which would imply that CSF is a collection of pathways) or, alternatively, whether they all converge on Erp1/Emi2 (which would imply that Erp1/Emi2 could be considered the molecular counterpart of CSF) (see Fig. 4).

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