Constant regulation of both the MPF amplification loop and the Greatwall-PP2A pathway is required for metaphase II arrest and correct entry into the first embryonic cell cycle

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Summary
Recent results indicate that regulating the balance between cyclin-B–Cdc2 kinase, also known as M-phase-promoting factor (MPF), and protein phosphatase 2A (PP2A) is crucial to enable correct mitotic entry and exit. In this work, we studied the regulatory mechanisms controlling the cyclin-B–Cdc2 and PP2A balance by analysing the activity of the Greatwall kinase and PP2A, and the different components of the MPF amplification loop (Myt1, Wee1, Cdc25) during the first embryonic cell cycle. Previous data indicated that the Myt1-Wee1-Cdc25 equilibrium is tightly regulated at the G2-M and M-G1 phase transitions; however, no data exist regarding the regulation of this balance during M phase and interphase. Here, we demonstrate that constant regulation of the cyclin-B–Cdc2 amplification loop is required for correct mitotic division and to promote correct timing of mitotic entry. Our results show that removal of Cdc25 from metaphase-II-arrested oocytes promotes mitotic exit, whereas depletion of either Myt1 or Wee1 in interphase egg extracts induces premature mitotic entry. We also provide evidence that, besides the cyclin-B–Cdc2 amplification loop, the Greatwall-PP2A pathway must also be tightly regulated to promote correct first embryonic cell division. When PP2A is prematurely inhibited in the absence of cyclin-B–Cdc2 activation, endogenous cyclin-A–Cdc2 activity induces irreversible aberrant mitosis in which there is, first, partial transient phosphorylation of mitotic substrates and, second, subsequent rapid and complete degradation of cyclin A and cyclin B, thus promoting premature and rapid exit from mitosis.

Key words: Greatwall, PP2A, Myt1, Wee1, Cdc25, Mitosis

Introduction
Entry and exit of mitosis are regulated by the cyclin-B–Cdc2 complex, also known as the M-phase-promoting factor (MPF). Mitotic entry is induced by the activation of this complex by phosphorylation (Nurse, 1990), whereas mitotic exit is promoted by inactivation through the ubiquitin-dependent degradation of its cyclin B regulatory subunit (Glotzer et al., 1991).

During G2, cyclin-B–Cdc2 is maintained in an inactive state by phosphorylation of the catalytic Cdc2 subunit on two inhibitory residues: threonine 14 and tyrosine 15 (Draetta and Beach, 1988; Gould and Nurse, 1989; Krek and Nigg, 1991). The identity of the kinases responsible for these phosphorylation events was first shown in yeast. Different genetic studies indicated that the protein kinases encoded by the genes wee1 and myt1 acted as negative regulators of cyclin-B–Cdc2 (Lundgren et al., 1991; Russell and Nurse, 1987a). Subsequent studies performed with human and Xenopus Wee1 and Myt1 proteins demonstrated a role for these two kinases in the phosphorylation of Thr14 and Tyr15 of Cdc2 (Mueller et al., 1995a; Mueller et al., 1995b; Parker and Piwnica-Worms, 1992). The subsequent activation of the cyclin-B–Cdc2 complex at mitotic entry is promoted by the dephosphorylation of these two inhibitory residues of Cdc2 by the phosphatase Cdc25 (Gautier et al., 1991; Kumagai and Dunphy, 1991; Millar et al., 1991; Strausfeld et al., 1991). At the end of G2, abrupt dephosphorylation of Tyr15 and Thr14 residues by Cdc25 triggers initial activation of cyclin-B–Cdc2, which in turn further activates Cdc25 and inactivates Wee1 and Myt1 by phosphorylation, resulting in full activation of the cyclin-B–Cdc2 complex (Hoffmann et al., 1993; Izumi et al., 1992; Mueller et al., 1995b; Russell and Nurse, 1987b; Smythe and Newport, 1992). This positive-feedback mechanism is called the MPF amplification loop.

Besides the direct regulation of cyclin-B–Cdc2 by this amplification loop, other feedback mechanisms also indirectly regulate cyclin-B–Cdc2 activation. In this regard, the Polo kinase Plx1 participates in the activation of cyclin-B–Cdc2 by directly phosphorylating and activating the Cdc25 phosphatase (Abrieu et al., 1998; Kumagai and Dunphy, 1996; Qian et al., 1998; Qian et al., 2001), and by promoting ubiquitin-dependent degradation of Wee1 (Watanabe et al., 2004) or inactivation of Myt1 (Nakajima et al., 2003).

The current model proposes that mitotic entry is promoted by cyclin-B–Cdc2 activation through the MPF amplification loop (Nurse, 1990), whereas mitotic exit is triggered by the inactivation of this complex through ubiquitin-dependent degradation of cyclin B (Murray et al., 1989). However, the involvement of other phosphatases in mitosis entry and exit has also been reported. In
this regard, it has been shown in interphase *Xenopus* egg extracts that the inhibition of PP2A by okadaic acid (OA) promotes cyclin-B–Cdc2 activation and blocks Cdc25 dephosphorylation, suggesting that PP2A negatively regulates MPF by maintaining Cdc25 in its inactive dephosphorylated form (Clarke et al., 1993; Felix et al., 1990). Accordingly, in fission yeast, decreased activity of PP2A induces premature mitosis, probably through the activation of Cdc25 (Kinoshita et al., 1990; Kinoshita et al., 1993). The role of PP2A in mitotic exit has also been suggested. Thus, inhibition of PP2A in *Xenopus* extracts from metaphase-II-arrested oocytes induces cyclin B degradation and exit from mitosis (Lorca et al., 1990). In addition, a decrease in PP2A activity promotes chromosome nondisjunction in fission yeast (Kinoshita et al., 1990). Moreover, a mutant CDC55 in budding yeast, which encodes a PP2A regulatory subunit, displays premature separation of sister chromatids and mitotic exit in the presence of an active spindle-assembly checkpoint (Minsull et al., 1996). Mitotic exit in this mutant takes place in the absence of cyclin B degradation through the inactivation of Cdc28 on its inhibitory residues. Finally, the activation of Cdc14 is also required in budding yeast to promote cyclin-B–Cdc2 substrate dephosphorylation and mitotic exit (D’Amours and Amon, 2004; Stegmeier and Amon, 2004). However, the Cdc14 phosphatase does not play a major role in late mitosis in fission yeast or in higher eukaryotes (Trautmann and McCollum, 2002).

Recent results have shed light on the mechanisms by which PP2A can control mitotic entry and exit (Castillo et al., 2009; Mochida et al., 2009; Vigneron et al., 2009). These new data indicate that PP2A induces dephosphorylation of MPF substrates. To promote mitotic entry and to maintain the mitotic state, this phosphatase must be inhibited by the recently identified Greatwall kinase. At mitotic exit, however, PP2A must be activated again to promote cyclin-B–Cdc2 substrate dephosphorylation (Castillo et al., 2009; Vigneron et al., 2009). Thus, it appears that regulating the balance between the cyclin-B–Cdc2 kinase and PP2A phosphatase is crucial to permit correct mitotic entry and exit.

In this work, we studied the regulation mechanisms controlling the balance between cyclin-B–Cdc2 and PP2A by analysing the activity of Greatwall and PP2A, and the different components of the MPF amplification loop at mitosis entry and during the first embryonic cell cycle.

Our data demonstrate that a constant counterbalance between the inhibitory kinases Myt1 and Wee1 and the activating phosphatase Cdc25 is required during metaphase II to prevent meiotic exit and during interphase of the first embryonic cell cycle to promote correct mitotic entry. Moreover, we also show that tight regulation of PP2A activity by Greatwall is essential to ensure correct timing of the first embryonic division. When PP2A is prematurely inhibited in the absence of cyclin-B–Cdc2 activity, endogenous cyclin-A–Cdc2 promotes an aberrant mitosis-like state in which there is only partial transient phosphorylation of the different cyclin-B–Cdc2 substrates followed by complete degradation of cyclin A and cyclin B.

**Results**

**Constant activity of Cdc25 is required to maintain MPF activity in CSF extracts and in metaphase-II-arrested oocytes**

The inhibitory role of Wee1- and Myt1-dependent phosphorylation of cyclin-B–Cdc2 during G2 is well established. Similarly, the activation of cyclin-B–Cdc2 through the dephosphorylation of its inhibitory sites by Cdc25 at mitotic entry has been largely described. However, less is known about the role of Cdc25, Myt1 and Wee1 in maintaining cyclin-B–Cdc2 activity during mitosis. Because only residual activity of Myt1 and/or Wee1 is present during mitosis (Smythe and Newport, 1992; Solomon et al., 1990), we asked whether Cdc25 is still required to maintain MPF activity in this phase of the cell cycle. To answer this question, we used egg extracts obtained from *Xenopus* oocytes arrested at metaphase II of meiosis (CSF extracts). Taking advantage of the fact that these extracts only contain the Cdc25C isoform, we could completely eliminate this phosphatase by immunodepletion and then analyse its effect on cyclin-B–Cdc2 activity. The depletion of Cdc25C induced complete dephosphorylation of the MPF substrates Erp1 (also called Emi2) and Cdc27, and a rapid decrease in cyclin-B–Cdc2 activity, as measured by histone H1 phosphorylation (Fig. 1A). We also observed dephosphorylation of cyclin B, rephosphorylation of Cdc2 on its inhibitory Tyr15 site and DNA decondensation. These results indicate that the extracts exited mitosis; however, this outcome occurred in the absence of cyclin B degradation. Moreover, we observed the same results when Cdc25 was depleted in CSF extracts in which the ubiquitin-dependent degradation pathway was blocked by the addition of the proteasome inhibitor MG132 (Fig. 1B) or when the anaphase-promoting complex (APC) was inhibited by Cdc27 depletion (data not shown). These results suggest that, rather than mitotic exit, Cdc25 depletion induced a loss of the mitotic state. This phenotype is specific to Cdc25 inhibition, as it was reversed by the addition of recombinant active Cdc25-GST protein to Cdc25-depleted CSF extracts (Fig. 1C). Although it seemed to be less efficient, the addition of anti-Cdc25 antibodies to CSF extracts induced the same effect, indicating that the simple addition of these antibodies blocks Cdc25 activity (Fig. 1D). Thus, we took advantage of the capacity of these antibodies to block Cdc25 to study the role of this phosphatase ‘in vivo’ in metaphase-II-arrested oocytes. We microinjected oocytes with either control (MI1+αCT) or anti-Cdc25 antibodies (MI1+αCdc25) in an MMR buffer supplemented with 5 mM EGTA to prevent oocyte activation. As shown in Fig. 1E, the microinjection of anti-Cdc25 antibodies induced dephosphorylation but not degradation of Erp1 (Emi2), and inactivation of cyclin-B–Cdc2, although this dephosphorylation occurred 30 minutes later in microinjected oocytes than in Cdc25-depleted CSF extracts. This indicates that the ‘in vivo’ inhibition of Cdc25 during metaphase II also induces the loss of the mitotic state. Thus, the activity of Cdc25 is continuously required to maintain the mitotic state ‘in vitro’ in CSF extracts and ‘in vivo’ in metaphase-II-arrested oocytes.

We subsequently analysed whether the inactivation of cyclin-B–Cdc2 induced by Cdc25 removal is dependent on the Myt1 and/or Wee1 kinases. We co-depleted Wee1 and Cdc25 from CSF extracts. As depicted in Fig. 2A, the co-depletion of Cdc25 and Wee1 abolished the phenotype observed by Cdc25 removal. Thus, under these conditions, Erp1 (Emi2) and Cdc27 remained phosphorylated, cyclin-B–Cdc2 had high kinase activity, Try15 of Cdc2 stayed dephosphorylated and DNA remained condensed.

We next co-depleted Myt1 and Cdc25. Unlike Wee1, the co-depletion of Myt1 and Cdc25 from CSF extracts did not prevent Erp1 (Emi2) and Cdc27 dephosphorylation, or rephosphorylation of Tyr15 of Cdc2. However, although we observed a clear decrease in cyclin-B–Cdc2 activity, residual cyclin-B–Cdc2 kinase activity remained for up to 90 minutes after depletion. This was probably sufficient to maintain the condensation state of DNA throughout the experiment (Fig. 2B). Thus, Wee1 and, to a lesser extent, Myt1
Fig. 1. Cdc25 activity is required to maintain an active MPF kinase in CSF extracts and metaphase-II-arrested oocytes. (A) Two successive 15-minute immunoprecipitations with control (CT) or anti-Cdc25 (ΔCdc25) antibodies were developed in 30 μl CSF extract, as described in Materials and Methods. 1 μl CSF extract and 1 μl supernatant were analysed by western blot to verify the immunodepletion of this protein. Time points of 1 μl supernatant were removed at 0, 30, 60 and 90 minutes after the second immunoprecipitation and used either for western blot to visualise Erp1 (Emi2), Cdc27, phospho-Tyr15 of Cdc2 (pTyr15) and cyclin B2 (cyB2), or for measuring cyclin-B–Cdc2 activity by histone H1 kinase assay (H1K). 1000-2000 sperm nuclei per microlitre was added to 10 μl supernatant after immunoprecipitation and incubated for 90 minutes. After fixation of the samples with 1% formaldehyde containing DAPI (1 μg/ml), 1 μl sample was used to visualise chromatin condensation by light microscopy (bottom). (B) CSF extracts were supplemented with 0.7 μl proteasome inhibitor MG132 (final concentration 1 mM) or the same volume of DMSO. 15 minutes later, they were depleted with anti-Cdc25 antibodies as in A. (C) CSF extracts were immunoprecipitated with anti-Cdc25 antibodies and were subsequently supplemented with either GST or GST-Cdc25 recombinant protein. Samples were removed at the indicated time points to study the phosphorylation pattern of Erp1 (Emi2), Cdc27 and Tyr15 of Cdc2. Sperm nuclei were added to immunodepleted Cdc25 CSF extracts after the addition of GST or the GST-Cdc25 recombinant protein, and DNA condensation was analysed by light microscopy. (D) CSF extracts were incubated for 5 minutes with control (αCT) or anti-Cdc25 antibodies (αCdc25). Subsequently, samples were removed to perform western blots and to analyse DNA condensation. (E) Metaphase-II-arrested oocytes were microinjected with either control (MII+αCT) or anti-Cdc25 antibodies (MII+αCdc25) in an MMR buffer (see Materials and Methods) supplemented with 5 mM EGTA to prevent oocyte activation by microinjection. One oocyte was lysed by time point and used to analyse the phosphorylation pattern of Erp1 (Emi2) by western blot. Activated oocytes were obtained by microinjecting buffer in the presence of an MMR buffer without EGTA. Cyclin-B–Cdc2 activity was measured in non-injected (MII), activated (activated oocytes), anti-Cdc25 (α25) and control-injected (αCT) oocytes 90 minutes after microinjection by H1 kinase assay (H1K). Scale bars: 5 μm.
must be continuously counterbalanced by Cdc25 for the mitotic state to be maintained in metaphase II.

Constant activity of Myt1 and Wee1 is required to counterbalance Cdc25 activity during interphase, ensuring correct timing of first embryonic mitosis

The role of the inhibitory phosphorylation of Cdc2, in particular by the Wee1 kinase, during interphase of the first embryonic cell cycle has been well established (Murakami and Vande Woude, 1998; Walter et al., 2000). In this regard, Wee1-dependent phosphorylation of Cdc2 prevents premature entry into mitosis, allowing completion of meiosis II. However, unlike Wee1, no data exist regarding the activity of the Cdc25 phosphatase during this phase of the cell cycle. We analysed this issue using interphase Xenopus egg extracts arrested 50 minutes after ionophore addition. Interphase extracts contain high cyclin A and cyclin B levels. Cyclin A association with Cdc2 forms an active cyclin-A–Cdc2 complex that is not regulated by Thr14 or Tyr15 phosphorylation (Clarke et al., 1992). However, despite the fact that cyclin-A–Cdc2 is fully active after cyclin A synthesis, mitotic substrates remain dephosphorylated (Fig. 3A, Cdc27), indicating that cyclin-A–Cdc2 kinase cannot induce mitotic entry in the absence of cyclin-B–Cdc2. Unlike cyclin-A–Cdc2, cyclin-B–Cdc2 is rapidly inactivated by Myt1 and Wee1 (note phosphorylation of Cdc2 on inhibitory Tyr15 site in Fig. 3A, time 0 minutes). To investigate whether Cdc25 activity was present in these extracts, we depleted the two inhibitory kinases Wee1 and Myt1. We then analysed by western blot the phosphorylation of the mitotic substrate Cdc27, the levels of cyclins A and B, and the activity of the cyclin-B–Cdc2 complex. As shown in Fig. 3A, we did not observe any phosphorylation of the mitotic substrate Cdc27; however, we clearly detected degradation of cyclins A and B concomitant with loss of the inhibitory phosphorylation on Tyr15 of Cdc2. From these results, we hypothesised that depletion of these two inhibitory kinases would induce activation of the cyclin-B–Cdc2 complex, which would subsequently trigger degradation of cyclin A and cyclin B, thus mimicking normal mitotic entry and exit.

To investigate whether this was the case, we depleted the extracts of either Wee1 (Fig. 3B) or Myt1 (Fig. 3C), and performed a time-course analysis of the phosphorylation of the mitotic substrate Cdc27, the levels of cyclins A and B, and the inhibitory phosphorylation on Tyr15 of Cdc2. The results demonstrate that the depletion of either of these two kinases induces rapid phosphorylation of Cdc27 concomitant with cyclin-B–Cdc2 activation (note dephosphorylation of Tyr15 of Cdc2), followed by the degradation of cyclin A and cyclin B. Depletion of Wee1 kinase induces quicker activation of cyclin-B–Cdc2 than Myt1 depletion, indicating that the former probably participates to a greater extent than the latter in the inhibition of this complex. We next tested whether cyclin A and B proteolysis is dependent on the APC, as in normal mitosis. We analysed the effect of the co-depletion of Cdc27 and Wee1 on the activation of cyclin-B–Cdc2, and the degradation of cyclin A and B. As depicted in Fig. 3D, the concomitant removal of Cdc27 and Wee1 induced rapid entry into mitosis. Thus, 20 minutes after removal, cyclin B2 was phosphorylated whereas Try15 of Cdc2 was completely dephosphorylated. Consequently, the mitotic substrate Greatwall was phosphorylated at this time. However, unlike the sole depletion of Wee1, the double depletion of Cdc27 and Wee1 did not induce the proteolysis of either cyclin A or cyclin B. Interestingly, because of the stabilisation of these proteins, the mitotic state was maintained throughout the experiment, as shown by the condensed DNA present 60 minutes after depletion. Similar results were observed when Myt1 (instead of Wee1) was co-depleted with Cdc27 (Fig. 3E), although, as observed in Fig. 3C, the activation of cyclin-B–Cdc2 was delayed in this case. Finally, we analysed whether mitotic entry induced by Wee1 and/or Myt1 depletion is specific to the loss of these kinases. We performed a first depletion of Cdc27 in interphase egg extracts, followed by a second depletion of Wee1 or Myt1, and subsequently added recombinant GST-Wee1 or GST-Myt1, respectively. Samples were then taken every 20 minutes to analyse the mitotic state (Fig. 3F,G). Adding Wee1 and Myt1 to depleted interphase extracts prevented mitotic entry,
indicating that this effect is specific to the loss of these kinases. Because the simple depletion of Wee1 or Myt1 induces activation of cyclin-B–Cdc2, it is likely that Cdc25 is at least partially active in these extracts and that this activity is sufficient to induce mitotic entry and exit when either of the inhibitory kinases (Wee1 or Myt1) is removed. This is in fact the case; the double depletion of Cdc25 and Wee1 (Fig. 3H) or Myt1 (data not shown) completely reverses the Wee1- or Myt1-depletion phenotype, indicating that mitotic entry is mediated by Cdc25. It is noteworthy that this phosphatase showed higher electrophoretic mobility in interphase compared to mitotic extracts (Fig. 3H), suggesting either that only an undetectable part of this phosphatase is phosphorylated during interphase or that the non-shifted form corresponds to a partially active phosphatase.

These results indicate that, in interphase of the first embryonic cell cycle, Myt1 and Wee1 must both be active to counterbalance the partial activity of Cdc25. The sole removal of one of these kinases is sufficient to induce first mitotic entry followed by subsequent mitotic exit. This removal promotes rapid activation of cyclin-B–Cdc2 kinase, which induces entry into mitosis by
phosphorylation of the different mitotic substrates, among them the APC subunit Cdc27. The phosphorylation of Cdc27 probably activates the APC, triggering degradation of cyclin A and cyclin B and mitotic exit.

**Mitotic entry induced by Wee1 or Myt1 depletion in interphase extracts is dependent on cyclin-A–Cdc2 and Plx1 kinases**

The fact that the removal of Wee1 and Myt1 induces the activation of cyclin-B–Cdc2 implies that partial activity of Cdc25 is already present in these extracts. Because Plx1 has been largely described as an activator of Cdc25 (Kumagai and Dunphy, 1996; Qian et al., 1998; Qian et al., 2001), we asked whether Plx1 might be required to maintain this partial activity of Cdc25 and to promote the activation of cyclin-B–Cdc2 after Wee1 and Myt1 co-depletion. To test this hypothesis, we performed a triple depletion of Wee1, Myt1 and Plx1 in interphase extracts. As shown in Fig. 4A, unlike the double removal of Wee1 and Myt1 (Fig. 3A), the co-depletion of Plx1, Wee1 and Myt1 did not induce cyclin-B–Cdc2 activation, because tyrosine 15 of Cdc2 remained phosphorylated, cyclin A and cyclin B were stable, and Cdc27 was maintained in a dephosphorylated state. This effect is specific to Plx1 depletion, because cyclin-B–Cdc2 is reactivated after Plx1-Wee1-Myt1 depletion if a constitutively active form but not a kinase-dead form of Plx1 is added to these depleted extracts (Fig. 4B). Moreover, the reactivation of this complex is also observed if these extracts were first co-depleted of Plx1, Wee1 and Myt1, and subsequently supplemented with an ectopic recombinant Cdc25 protein (Fig. 4C). These results indicate that Plx1 is required to induce cyclin-B–Cdc2 activation and mitotic entry through the activation of Cdc25.

Previously reported data indicate that the level of Plx1 activity increases from 20 to 40 minutes after oocyte activation and that this increase is concomitant with the increase in cyclin-A–Cdk activity (Abrieu et al., 1998) (our unpublished results). Moreover, previous data from starfish oocytes demonstrate a role for cyclin-
A–Cdc2 in Plk1 and cyclin-B–Cdc2 activation during the first embryonic division (Okano-Uchida et al., 2003). To test whether cyclin-A–Cdc2 could participate in the activation of Plx1 in Xenopus oocytes, we either depleted Plx1 or did not and next added purified cyclin A to cycloheximide-treated interphase egg extracts, which are devoid of endogenous cyclin B and cyclin A. We analysed cyclin-A–Cdc2 and Plx1 activity, as well as Cdc25 phosphorylation. As depicted in Fig. 4D, the addition of ectopic cyclin A promotes the activation of Plx1. However, despite the fact that cyclin-A–Cdc2 activity was identical in non-depleted and Plx1-depleted extracts, the phosphorylation of Cdc25 was only observed when Plx1 was present. These results indicate that, in these extracts, cyclin-A–Cdc2 induces Cdc25 phosphorylation through the activation of Plx1.

We further investigated whether endogenous cyclin-A–Cdc2 is required to maintain partial Cdc25 activity by co-depleting interphase extracts of cyclin A, Myt1 and Wee1. Under these conditions, no activation of cyclin-B–Cdc2 was observed, phosphorylation on Try15 of Cdc2 was maintained, Cdc27 was dephosphorylated and cyclin B2 remained stable (Fig. 4E). Thus, cyclin-A–Cdc2 activity, probably through the activation of Plx1, is essential to maintain the partial activity of Cdc25, which promotes the activation of cyclin-B–Cdc2 after Wee1 and Myt1 depletion.

Together, these results indicate that the balance between Wee1 and Myt1 kinases and Cdc25 phosphatase is tightly regulated in metaphase II and in interphase of first mitosis to ensure correct first embryonic cell division.

**Besides Wee1, Myt1 and Cdc25, regulation of PP2A is also essential to maintain the correct timing of mitosis in the first embryonic cell cycle**

Recent results demonstrate that the inhibition of the PP2A phosphatase by the Greatwall kinase is essential for inducing the correct timing of mitosis (Castilho et al., 2009; Vigneron et al., 2009). Depletion of Greatwall in CSF extracts induces the loss of the mitotic state, indicating that, similar to Cdc25, it is required to maintain mitosis. In addition, Greatwall is also required for entry into mitosis. In this regard, it has been shown that the depletion of Greatwall from cycling extracts prevents mitotic entry (Yu et al., 2006). We asked whether Greatwall is also required to induce mitotic entry and exit in interphase extracts after the removal of Wee1 and Myt1. We co-depleted these three proteins and analysed whether cyclin-B–Cdc2 was activated under these conditions. The removal of Greatwall prevents mitotic entry in these extracts (Fig. 5A). This is a specific effect of Greatwall depletion, because adding this protein completely rescued this phenotype (Fig. 5B). Moreover, the loss of mitotic entry is due to the persistently high PP2A activity, because its inhibition by the addition of microcystin completely reversed this phenotype (Fig. 5C). We conclude that Greatwall-dependent PP2A inhibition is required for the partial activity of Cdc25 to promote cyclin-B–Cdc2 activation after the removal of the Wee1 and Myt1.

**Fig. 5. The regulation of phosphatases is also essential to maintain correct timing of mitosis in the first embryonic cell cycle.** (A) Interphase extracts were depleted of Greatwall (GW), Myt1 and Wee1, and mitotic entry was determined by analysing the phosphorylation of the indicated proteins. The efficacy of anti-Greatwall antibodies to immunodeplete this protein is shown. (B) Interphase extracts were depleted of Greatwall and Cdc27 proteins. The supernatants were then supplemented with GST-Greatwall recombinant protein and immunoprecipitated again with anti-Wee1 and anti-Myt1 antibodies to deplete these two proteins. Mitotic entry was then analysed by western blot. The levels of endogenous and recombinant GST-Greatwall proteins are shown. (C) Similar to A, except for the addition of 800 nM of the PP2A inhibitor microcystin (Mycs) after protein depletion. (D) Interphase extracts were first depleted of cyclin A and subsequently supplemented with 800 nM microcystin. (E) Interphase extracts were immunoprecipitated with anti-cyclin A and anti-Cdc25 antibodies, and subsequently supplemented with 800 nM microcystin. Mitotic entry was analysed by western blot. Scale bar: 5 μm.
kinases. These results were expected, because the sole addition of microcystin to interphase extracts promotes cyclin-B–Cdc2 activation and mitotic entry (Felix et al., 1990).

We showed above that Cdc25 has partial activity that is counterbalanced by Wee1 and Myt1 in interphase extracts. This activity is probably required to induce robust activation of the MPF amplification loop and to promote rapid mitotic entry. We next asked whether this partial activity was still required to promote cyclin-B–Cdc2 activation when PP2A was inhibited. To do that, we prevented the partial activation of Cdc25 by depletion of cyclin A and subsequently inhibited PP2A by the addition of microcystin. The removal of cyclin A did not prevent either MPF activation or degradation of cyclin B after PP2A inhibition (Fig. 5D). Thus, the activation of Cdc25 dependent on cyclin-A–Cdc2 and Plx1 is not required when mitotic-substrate dephosphorylation was prevented by PP2A inhibition. However, cyclin-B–Cdc2 activation was still dependent on Cdc25, because no activation was observed when the co-depletion of cyclin A and Cdc25 was performed (Fig. 5E).

It is well established that most of the cyclin-B–Cdc2 substrates can also be phosphorylated in vitro by cyclin-A–Cdc2. We showed above that, in interphase extracts, cyclin-A–Cdc2 activity is not sufficient to phosphorylate all of the mitotic substrates by itself or to promote proteolysis (see Fig. 3H). Rather, this activity seems to be required to maintain partial Plx1-dependent activation of Cdc25 (see Fig. 4A,E). However, the endogenous cyclin-A–Cdc2 activity could be sufficient to phosphorylate the mitotic substrates under PP2A inhibition. We investigated this possibility in interphase extracts in which the activation of cyclin-B–Cdc2 was prevented by Cdc25 depletion and PP2A was inhibited by microcystin. The addition of microcystin to these extracts promoted phosphorylation of the mitotic substrates Greatwall and Cdc27, and degradation of cyclin A and cyclin B (Fig. 6A). We also observed a late dephosphorylation on Tyr15 of Cdc2. We reasoned that this dephosphorylation could be the result of the degradation of cyclin B. However, we could not exclude the fact that activation of cyclin-B–Cdc2 could be triggered by incomplete depletion of Cdc25. To exclude this possibility, we repeated the same experiment by co-depleting Cdc27 and Cdc25 to block cyclin B proteolysis. As depicted in Fig. 6B, phosphorylation of Greatwall was observed, although, in this case, Try15 of Cdc2 stayed phosphorylated, indicative of an inactive cyclin-B–Cdc2 (Fig. 6B). Thus, the endogenous activity of cyclin-A–Cdc2 can phosphorylate at least some of the mitotic substrates of Cdc2 and induce proteolysis of cyclin A and cyclin B when PP2A is inhibited. To investigate whether the other substrates of cyclin-B–Cdc2 could also be phosphorylated by endogenous cyclin-A–Cdc2, we analysed the general cyclin-B–Cdc2-dependent phosphorylation state in these extracts using an antibody directed against the phosphorylated serine of the Cdk consensus motif. A strong signal, corresponding to phosphorylated MPF substrates, was observed when cyclin-B–Cdc2 is present in the microcystin-treated extracts independently of the presence or absence of cyclin-A–Cdc2 (Fig. 6C, Mycs and ΔcyA). However, this signal was clearly lower when only cyclin-A–Cdc2 was left in these extracts (Fig. 6C, Δcyc25). These results indicate that, despite the fact that cyclin-A–Cdc2 can induce phosphorylation of some cyclin-B–Cdc2 substrates (such as Greatwall or Cdc27) and thus the degradation of cyclin A and cyclin B, this complex cannot promote normal mitosis because most of the mitotic substrates are not correctly phosphorylated.

**Discussion**

The role of Cdc25 in promoting cyclin-B–Cdc2 activation and mitotic entry during oocyte maturation and the first embryonic cell cycle has been largely demonstrated (Gautier et al., 1991; Kumagai and Dunphy, 1991). Similarly, the inhibitory role of Myt1 and Wee1 towards Cdc2 to prevent mitotic entry during interphase of the first mitotic division is well established (Mueller et al., 1995a; Mueller et al., 1995b). However, there are no data on whether the regulatory balance between the Myt1 and Wee1 kinases and Cdc25 phosphatase is continuously working throughout the cell cycle or whether it is limited just to the boundary of cyclin-B–Cdc2 activation. The major mechanism that induces MPF inactivation upon mitotic exit is cyclin B degradation; thus, we would expect that, once cyclin-B–Cdc2 is activated, the Myt1–Wee1–Cdc25 loop is no longer required. We could also imagine that Cdc25 is not active during interphase and that only the activities of Myt1 and Wee1 are present to maintain inhibition of cyclin-B–Cdc2. However, surprisingly we demonstrated the presence of partial activity of Wee1 and Myt1 during metaphase II arrest and of Cdc25 during interphase of the first embryonic cell cycle, indicating that a constant counterbalance between the inhibitory kinases and the activating phosphatase is present during this first embryonic cell division. During metaphase II arrest, *Xenopus* oocytes show...
partial but constant degradation of cyclin B, which is counterbalanced by new synthesis of this protein (Yamamoto et al., 2005). This implies the formation of new cyclin-B–Cdc2 complexes, which could be partially phosphorylated and inhibited by Myt1 and Wee1 kinases to accurately control a constant level of cyclin-B–Cdc2 activity during metaphase II. Besides the role of the amplification loop in maintaining cyclin-B–Cdc2 activity during metaphase II, this pathway must also be important during interphase of the first embryonic cell cycle, in which there are no G1-G2 phases and additional gaps of time are required to complete meiosis II (polar body extrusion, decondensation of sperm nucleus and fusion of the two pronuclei). The constant functionality of this loop would ensure that these gaps occur and would confer the rapid subsequent resumption of the cell cycle required for correct embryonic development. This loop also seems to be functional during G2 in fission yeast. Accordingly, Wee1 mutants initiate mitosis at half the cell size of the wild type, whereas the overexpression of this kinase delayed mitosis until cells grow to a larger size (Russell and Nurse, 1987a). We do not know whether this feedback is also constantly active during the mitotic cell cycle in somatic cells; however, this seems to be the case because some data have recently demonstrated that the inhibitory kinases Wee1 and Myt1 have the capacity to negatively regulate Cdc2 activity in G1 (Potapova et al., 2009).

Apart from the activation of cyclin-B–Cdc2, the inhibition of the PP2A phosphatase is also essential for correct timing of mitosis. In this regard, it has been shown in both *Xenopus* egg extracts and fission yeast that the inhibition of PP2A induces premature mitotic entry and that this effect is correlated with Cdc25 hyperphosphorylation and activation (Clarke et al., 1993; Felix et al., 1990; Kinoshita et al., 1990; Kinoshita et al., 1993). However, the activity of this phosphatase seems to be required again to promote mitotic exit (Kinoshita et al., 1990; Minshull et al., 1996). According to these data, it has been recently demonstrated that two different kinase activities, cyclin-B–Cdc2 and Greatwall, are essential for mitotic entry, the former to phosphorylate mitotic substrates and the latter to prevent massive dephosphorylation of these substrates by PP2A (Castillo et al., 2009; Vigneron et al., 2009). At mitotic entry, Greatwall inhibits PP2A, the phosphatase capable of dephosphorylating the majority of cyclin-B–Cdc2 substrates; at mitotic exit, Greatwall is inactivated, allowing the subsequent activation of PP2A and promoting cyclin-B–Cdc2 substrate dephosphorylation. In this way, we asked whether Greatwall and the inactivation of PP2A are also required to correctly develop the first mitotic cell cycle. Interestingly, we show that this is the case.

As described above, in the absence of the inhibitory activity of Myt1 and/or Wee1, partial activity of Cdc25 maintained by cyclin-A–Cdc2 and Plx1 is required to induce mitotic entry in interphase. However, our results demonstrate that the inhibition of PP2A induces mitotic entry, even in the absence of activation of Cdc25 dependent on cyclin-A–Cdc2 and Plx1. Thus, neither cyclin-A–Cdc2 nor Plx1 are required to activate cyclin-B–Cdc2 when PP2A is inhibited. A regulatory relationship between Polo and Greatwall has previously been suggested in *Drosophila* (Archambault et al., 2007). *Drosophila* females with the Scant mutation, a hyperactive form of Greatwall, show enhanced female sterility induced by *polo* mutants. These data suggest that Greatwall antagonizes Polo (Archambault et al., 2007). However, our results clearly demonstrate an agonistic relationship between these two proteins. We do not have an explanation for the origin of these differences between the two models; however, it is possible that they might reflect different aspects of the mechanisms regulating cell-cycle progression.

It is likely that, in the absence of activation of Cdc25 dependent on cyclin-A–Cdc2 and Plx1, the small amount of cyclin-B–Cdc2 complex that could be activated by the basal activity of Cdc25 would promote partial phosphorylation of Wee1 and Myt1, favouring still more the activation of the cyclin-B–Cdc2 complex and thus triggering the MPF amplification loop. In this regard, the complete removal of cyclin-A–Cdc2 and cyclin-B–Cdc2 prevents mitotic entry after PP2A inhibition, indicating that kinase activity of either cyclin-A–Cdc2 or cyclin-B–Cdc2, or both, is required to promote mitotic entry, even without PP2A activity. Interestingly,
after PP2A inhibition and in the absence of cyclin-B–Cdc2 activity, the endogenous cyclin-A–Cdc2 is sufficient to promote phosphorylation of some mitotic substrates, such as Cdc27 and Greatwall, and to trigger cyclin A and cyclin B degradation. However, the general cyclin-B–Cdc2-dependent phosphorylation pattern in these extracts indicates a clear decrease in the phosphorylation level of the majority of the mitotic substrates, suggesting that the activity of cyclin-A–Cdc2 under these conditions is not sufficient to induce correct mitosis. Thus, PP2A activity must be tightly regulated to allow proper mitosis, because the premature inhibition of PP2A in interphase at a moment when cyclin A but not cyclin B is completely accumulated would probably induce poor phosphorylation of the majority of mitotic substrates, but would activate the APC. This would then degrade cyclin A and cyclin B, promoting an abnormal and irreversible first mitotic division.

Our results could be explained by the model depicted in Fig. 7. We propose that, during interphase, cyclin A and cyclin B accumulate, giving an active cyclin-A–Cdc2 complex but a Wee1- and Myt1-inhibited MPF complex. The accumulation of active cyclin-A–Cdc2 will induce partial activation of Plx1, which, in turn, partially activates Cdc25. This partially active Cdc25 will promote a burst of MPF activity, which will phosphorylate and inhibit Myt1 and Wee1 and further activate Cdc25, triggering the MPF amplification loop. However, the complete activation of MPF is not sufficient to induce mitotic phosphorylation in the presence of high PP2A activity (Vigneron et al., 2009), unless Greatwall is activated. It is the activation of Greatwall by MPF itself that will finally promote mitotic entry.

According to this model, when cyclin-B–Cdc2 accumulates during interphase to a certain level, Myt1 and/or Wee1 are no longer capable of completely phosphorylating MPF and thus, the partial activity of Cdc25 maintained by cyclin-A–Cdc2 and Plx1 will be sufficient to trigger the MPF amplification loop (Fig. 3). However, this will not be the case if we prevent Cdc25 partial activation by inhibiting either of these two kinases (Fig. 4). On the other hand, if PP2A is inhibited, the basal activity of Cdc25 would be sufficient to completely activate MPF, even in the absence of cyclin-A–Cdc2 or Plx1 (Fig. 5). Finally, if we artificially decrease the dephosphorylation of mitotic substrates by the inhibition of PP2A in the absence of cyclin-B–Cdc2 complex, partial phosphorylation of some mitotic substrates will be performed. One of these proteins is the APC subunit Cdc27, which, once phosphorylated, will promote APC activation and degradation of cyclin A and B (Fig. 6).

In conclusion, our results demonstrate that, during the first mitotic division, the MPF amplification loop is constantly active throughout the cell cycle. This activity is probably required to promote correct timing by providing pseudo-gaps during this first division without affecting rapid mitotic resumption after meiosis II completion. Moreover, we also demonstrate that the correct timing by providing pseudo-gaps during this first mitotic division is not sufficient to induce correct mitosis. Thus, PP2A activity must be tightly regulated to allow proper mitosis, because the premature inhibition of PP2A in interphase at a moment when cyclin A but not cyclin B is completely accumulated would probably induce poor phosphorylation of the majority of mitotic substrates, but would activate the APC. This would then degrade cyclin A and cyclin B, promoting an abnormal and irreversible first mitotic division.

Materials and Methods

Immunoassays: protein purification and antibodies
Anti-Greatwall and anti-Wee1 antibodies were obtained as previously described (Vigneron et al., 2009). Anti-Myt1 antibodies were generated against a peptide (H2N-CRNLKGMDAATQ-COOH) corresponding to the C-terminal sequence of the Xenopus Myt1 protein. Peptides were coupled to thyroglobulin for immunisation and to immobilised BSA for affinity purification, as previously described (Lorca et al., 2001).

Polyclonal phospho-Tyr-15 Cdc2 and anti-phospho-Ser Cdk substrates were obtained from Cell Signalling Technology. Affinity-purified antibodies against Cdc27, cyclin B2, Cdc25, Plx1 and Epl1 (Emi2) were obtained as previously described (Abrieu et al., 1998; Bernis et al., 2007; Castro et al., 2001; Lorca et al., 1998).

Recombinant human Cdc25B-MBP was a generous gift of Veronique Baldin (CRSM, CNRS, Montpellier, France).

Full-length human cyclin A was produced in Escherichia coli and purified as previously reported (Lorca et al., 1992).

Preparation of Xenopus egg extracts and sperm nuclei, and immunoprecipitation

CSF egg extracts were prepared from unfertilised Xenopus eggs that were arrested at metaphase of the second meiotic division, as previously described (Murray and Kirschner, 1991). Interphase egg extracts and cycloheximide-treated interphase extracts were prepared from dejellified unfertilised eggs transferred in MMR/4 (25 mM NaCl, 0.5 mM KCl, 0.25 MgCl2, 0.025 mM NaEGTA, 1.25 mM HEPES-NaOH pH 7.7) supplemented with cycloheximide (100 µg/ml) or left untreated. Extracts were prepared 50 minutes after ionophore addition by the same procedure as described for CSF extracts.

Demembranated sperm nuclei were prepared as described (Glotzer et al., 1991). Immunoprecipitations and immunodepletions were performed using 10 µl extract, 10 µl magnetic protein G-Dynabeads (Dynal) and 2 µg each antibody. Antibody-linked beads were washed two times with RIPA (10 mM NaH2PO4, 100 mM NaCl, 5 mM EDTA, 1% Triton X100, 0.5% deoxycholate, 80 µM β-glycerophosphate, 50 mM NaF, 1 mM DTT), two times with 30 mM TRIS pH 7.5 and incubated for 15 minutes at room temperature with 10 µl Xenopus egg extracts. For immunodepletion, the supernatant was recovered and used for subsequent experiments. When two subsequent immunodepletions were performed, the supernatant from the first immunodepletion was recovered and used for the second one. Two consecutive immunoprecipitations were made to completely remove the endogenous Cdc25, Plx1, Greatwall and Myt1 proteins, whereas one immunoprecipitation was enough to completely deplete endogenous Wee1, Cdc27 and cyclin A.

H1 kinase and casein kinase assays

Extract (1 µl) was frozen in liquid nitrogen at the indicated times. Extract samples were then thawed by the addition of 19 µl 1 H buffer, including [γ-32P]ATP (Zhang et al., 1997), and incubated for 10 minutes at room temperature. Reactions were stopped by adding Laemmli sample buffer and analysed by SDS-PAGE.

Casein kinase assays were performed as previously described (Abrieu et al., 1998).

Light microscopy

A DMR A Leica microscope DM 4500B with a 63× immersion oil objective (HCX PLAPO), tube factor 1 was used for epifluorescence imaging. Images were captured with a CoolSnap HQ camera (Roget Scientific) and the whole set was driven by MetaMorph (Universal Imaging, Downingtown, PA).

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References

Abrieu, A., Brassac, T., Galas, S., Fisher, D., Labbe, J. C. and Doree, M. (1998). The Polo-like kinase Plx1 is a component of the MPF amplification loop at the G2/M-phase transition of the cell cycle in Xenopus eggs. J. Cell Sci. 111, 1751-1757.

Archambault, V., Zhao, X., White-Cooper, H., Carpenter, A. T. and Glover, D. M. (2007). Mutations in Drosophila Greatwall/Scnt reveal its roles in mitosis and meiosis and interdependence with Polo kinase. PLoS Genet. 3, e280.

Bernis, C., Vigneron, S., Burgess, A., Labbe, J. C., Fesquet, D., Castro, A. and Lorca, T. (2007). Pin1 stabilizes Emil during G2 phase by preventing its association with SCF(betacaten). EMBO Rep. 8, 91-98.

Castillo, P. V., Williams, B. C., Mischida, S., Zhao, Y. and Goldberg, M. L. (2009). The M phase kinase Greatwall (Gwl) promotes inactivation of PP2A/B55delta, a phosphatase directed against CDK phosphosites. Mol. Biol. Cell 20, 4777-4789.

Castro, A., Peter, M., Magnaghi-Jaulin, L., Vigneron, S., Galas, S., Lorca, T. and Labbe, J. C. (2001). Cyclin B/cdc2 induces c-Mos stability by direct phosphorylation in Xenopus oocytes. Mol. Biol. Cell 12, 2660-2671.

Clarke, P. R., Leiss, D., Pagano, M. and Karsenti, E. (1992). Cyclin A- and cyclin B-dependent protein kinases are regulated by different mechanisms in Xenopus egg extracts. EMBO J. 11, 1751-1761.

Clarke, P. R., Hoffmann, I., Draetta, G. and Karsenti, E. (1993). Dephosphorylation of cdc25-C by a type-2A protein phosphatase: specific regulation during the cell cycle in Xenopus egg extracts. Mol. Biol. Cell 4, 397-411.
D'Amours, D. and Amon, A. (2004). At the interface between signaling and executing anaphase-Cdc14 and the FEAR network. *Genes Dev.* 18, 2581-2595.

Draetta, G. and Beach, D. (1988). Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. *Cell* 54, 17-26.

Felix, M. A., Cohen, P. and Karsten, E. (1990). Cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the Xenopus early embryonic cell cycle: evidence from the effects of okadaic acid. *EMBO J.* 9, 675-683.

Gautier, J., Solomon, M. J., Booser, R. N., Bazan, J. F. and Kirschner, M. W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. *Cell* 67, 197-211.

Glotzer, M., Murray, A. W. and Kirschner, M. W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* 349, 132-138.

Gould, K. L. and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2 plus protein kinase regulates entry into mitosis. *Nature* 342, 39-45.

Hoffmann, I., Clarke, P. R., Marcote, M. J., Karsten, E. and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self-activation of MPF at mitosis. *EMBO J.* 12, 53-63.

Izumi, T., Walker, D. H. and Maller, J. L. (1992). Isolation of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J. Biol. Chem.* 278, 25277-25280.

Murakami, M. S. and Vande Woude, G. F. (1998). Activation of the early embryonic cyclin Cdc25; regulation of cell cycle length by Xe-wee1 and Mos. *Development* 125, 237-248.

Murray, A. W. and Kirschner, M. W. (1991). What controls the cell cycle? *Sci. Am.* 264, 56-65.

Murray, A. W., Solomon, M. J. and Kirschner, M. W. (1988). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* 339, 280-286.

Nakajima, H., Toyoshima-Morimoto, F., Taniguchi, E. and Nishida, E. (2003). Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J. Biol. Chem.* 278, 25277-25280.

Murakami, M. S. and Vande Woude, G. F. (1998). Activation of the early embryonic cyclin Cdc25; regulation of cell cycle length by Xe-wee1 and Mos. *Development* 125, 237-248.

Murray, A. W. and Kirschner, M. W. (1991). What controls the cell cycle? *Sci. Am.* 264, 56-65.

Murray, A. W., Solomon, M. J. and Kirschner, M. W. (1988). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* 339, 280-286.

Murakami, M. S. and Vande Woude, G. F. (1998). Activation of the early embryonic cyclin Cdc25; regulation of cell cycle length by Xe-wee1 and Mos. *Development* 125, 237-248.

Murray, A. W. and Kirschner, M. W. (1991). What controls the cell cycle? *Sci. Am.* 264, 56-65.

Murray, A. W., Solomon, M. J. and Kirschner, M. W. (1988). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* 339, 280-286.

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