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The polo-like kinase Plx1 is required for M phase exit and destruction of mitotic regulators in *Xenopus* egg extracts

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Polo-like kinases (Plks), named after the *Drosophila* gene product polo, have been implicated in the regulation of multiple aspects of mitotic progression, including the activation of the Cdc25 phosphatase, bipolar spindle formation and cytokinesis. Genetic analyses performed in yeast and *Drosophila* suggest a function for Plks at late stages of mitosis, but biochemical data to support such a function in vertebrate organisms are lacking. Here we have taken advantage of *Xenopus* egg extracts for exploring the function of Plx1, a *Xenopus* Plk, during the cell cycle transition from M phase to interphase (I phase). We found that the addition of a catalytically inactive Plx1 mutant to M phase-arrested egg extracts blocked their Ca^{2+}-induced release into interphase. Concomitantly, the proteolytic destruction of several targets of the anaphase-promoting complex and the inactivation of the Cdc2 protein kinase (Cdk1) were prevented. Moreover, the M to I phase transition could be abolished by immunodepletion of Plx1, but was restored upon the addition of recombinant Plx1. These results demonstrate that the exit of egg extracts from M phase arrest requires active Plx1, and they strongly suggest an important role for Plx1 in the activation of the proteolytic machinery that controls the exit from mitosis.

Keywords: mitosis/M phase/Plx1/polo-like kinase/*Xenopus*

Introduction

Polo-like kinases (Plks) are emerging as a new family of cell cycle regulators. Prominent members of the Plk family include *Drosophila melanogaster* polo, *Saccharomyces cerevisiae* Cdc5p, *Schizosaccharomyces pombe* $plo1^+$, mammalian Plk1 and *Xenopus laevis* Plx1 (Glover *et al*., 1996; Lane and Nigg, 1997). Genetic studies indicate that Plks contribute to promote various aspects of mitotic progression, but biochemical studies on the molecular action of these kinases remain scarce. In *Drosophila*, mutations in the *polo* gene result in a high frequency of cells with monopolar spindles and disorganized spindle poles (Sunkel and Glover, 1988). A similar phenotype has also been observed in *S.pombe*, where disruption of the $plo1^+$ gene results in a defect in bipolar spindle assembly (Ohkura *et al*., 1995). In mammalian cells, antibody microinjection experiments have revealed a function for Plk1 in centrosome maturation (Lane and Nigg, 1996). Additional support for an important function of Plks at the onset of mitosis has been obtained in *Xenopus laevis*, where Plx1 has been identified as a protein kinase able to phosphorylate and thereby activate the Cdc25 phosphatase, a positive regulator of Cdk1/cyclin B (M phase-promoting factor, MPF) (Kumagai and Dunphy, 1996). Taken together, these studies indicate that Plks may play important roles at the onset of mitosis.

Studies on the *CDC5* gene in *S.cerevisiae* have failed to reveal an essential role for this kinase prior to the onset of anaphase although, interestingly, Cdc5p appears to be required for adaptation to a DNA damage checkpoint at G_2/M (Toczyski *et al*., 1997). Instead, the analysis of *cdc5* mutant phenotypes suggests that this kinase is required late during mitosis, as both *cdc5 ts* and null mutant strains arrest with separated chromosomes and elongated spindles (Hartwell *et al*., 1973; Kitada *et al*., 1993). Similarly, $plo1^+$ of *S.pombe* may be important not only for spindle assembly but also for cytokinesis (Ohkura *et al*., 1995). Taken together, the available evidence thus suggests that Plks may function during multiple stages of mitosis (Glover *et al*., 1996; Lane and Nigg, 1997).

Progression through mitosis is regulated by two fundamental mechanisms, protein phosphorylation and degradation (Deshaies, 1995; Nigg, 1995; King *et al*., 1996). In particular, proteolysis has been identified as a key mechanism essential for both sister chromatid separation at the onset of anaphase and the inactivation of Cdk1 upon exit from mitosis. Proteins containing characteristic sequence motifs, termed destruction boxes, are targeted for proteolysis by cell cycle-dependent polyubiquitination (Glotzer *et al*., 1991). This modification is brought about by the sequential action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (reviewed in Hershko and Ciechanover, 1992; Varshavsky, 1997). Then, ubiquitinated proteins are degraded by the 26S proteasome (Jentsch and Schlenker, 1995). A large multiprotein complex with E3 activity specific for mitotic substrates has been purified from several species, including *S.cerevisiae* (Zachariae *et al*., 1996), the clam *Spisula* (Sudakin *et al*., 1995) and *Xenopus* (King *et al*., 1995). This complex consists of some 8–12 subunits and has been termed cyclosome (Sudakin *et al*., 1995) or APC (anaphase-promoting complex) (King *et al*., 1995).

The first proteins identified as critical targets for degradation during mitosis were the mitotic cyclins (Murray *et al*., 1989; Glotzer *et al*., 1991; Lorca *et al*., 1991; Gallant and Nigg, 1992; Surana *et al*., 1993). More recently, an important role for proteolysis in the initiation of sister chromatid separation has emerged. Inhibition of APC function either by substrate competition in *Xenopus* extracts (Holloway *et al*., 1993) or by mutations in APC subunits in yeast (Irniger *et al*., 1995) prevents

chromosome segregation. These early findings implied that proteins other than mitotic cyclins must be degraded to allow anaphase onset. In support of this view, APC-dependent degradation of the protein Pds1p was shown to be essential for anaphase onset in *S.cerevisiae* (Cohen-Fix *et al*., 1996; Shirayama *et al*., 1998), and similar data have been obtained for Cut2, a probable functional homologue of Pds1p in *S.pombe* (Funabiki *et al*., 1996). The fact that different APC substrates are degraded at different times during mitosis (Hunt *et al*., 1992; Sigrist *et al*., 1995) implies the existence of regulatory mechanisms that allow the APC to act on different substrates according to a precise temporal (and perhaps spatial) pattern. Several lines of evidence indicate that phosphorylation may represent a major mechanism for regulating APC activity (King *et al*., 1995; Lahav-Baratz *et al*., 1995; Sudakin *et al*., 1995; Grieco *et al*., 1996; Peters *et al*., 1996; Yamada *et al*., 1997), but detailed information on its regulation has not yet been reported.

Extrapolating from the phenotype of *cdc5* mutants in *S.cerevisiae*, and, to a lesser extent, data obtained from *Drosophila* and *S.pombe*, we expected that vertebrate Plks might play a role during exit from M phase. However, previous attempts at revealing such a role in vertebrate cells had failed to produce conclusive results (Lane and Nigg, 1996; Mundt *et al*., 1997). In this study, we therefore turned to *Xenopus* egg extracts, a favourite model system for biochemical studies on the M to I phase transition (Murray, 1991). Using both a dominant-negative approach and immunodepletion–reconstitution experiments, we show that the activity of Plx1 is absolutely required for the Ca^{2+}-induced transition of M phase-arrested extracts to interphase. In the absence of Plx1 activity, the destruction of multiple mitotic substrates is impaired, indicating that Plx1 activity is required for the activation of the degradation machinery in response to a Ca^{2+} signal. These findings reveal a new role for a vertebrate Plk in the exit from M phase, and they suggest that Plks may be important regulators of APC activity.

Results

Plx1 activity is cell cycle regulated

To study Plx1 in *Xenopus* egg extracts, we have prepared polyclonal antibodies against recombinant Plx1 produced in baculovirus-infected insect cells. Immunoblotting revealed that both cytostatic factor (CSF)-arrested (M phase) extracts (Murray, 1991) and interphase (I phase) extracts contain similar levels of Plx1 (Figure 1, lanes 1 and 2). This contrasts with the cyclic accumulation of mammalian Plk1 (Golsteyn *et al*., 1995) and budding yeast Cdc5p (Hardy and Pautz, 1996; Shirayama *et al*., 1998), but is likely to reflect differences in the regulation of the rapid embryonic cycles as compared with somatic cycles. Similar observations have been made previously for other cell cycle regulators, including cyclin E (Rempel *et al*., 1995; Hartley *et al*., 1996). The concentration of Plx1 in the extracts used here was estimated to be ~10 μg/ml (150 nM) (data not shown). Notably, Plx1 displayed a retarded migration in M phase extracts as compared with I phase extracts (Figure 1, lanes 1 and 2). As suggested by previous studies on *Drosophila* polo (Tavares *et al*., 1996) and human Plk1 (Hamanaka *et al*., 1995), this altered migration most likely reflects phosphorylation of Plx1 in M phase extracts (see also Mundt *et al*., 1997).

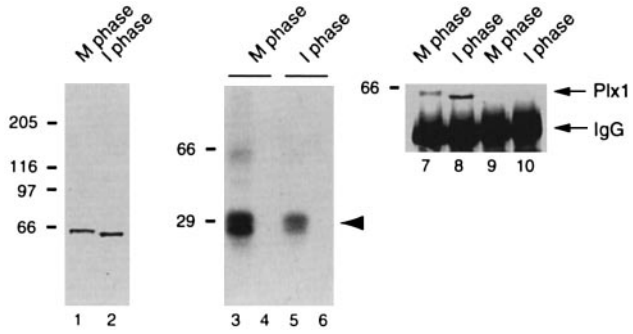

Fig. 1. Characterization of Plx1 expression and activity in *Xenopus* egg extracts. Plx1 was detected in M and I phase extracts by immunoblotting with anti-Plx1 antibodies (lanes 1 and 2). The activity of Plx1 was measured in immunoprecipitates prepared from M and I phase extracts (as indicated), using casein as a substrate (lanes 3–6; the arrow marks the migration of casein). Lanes 3 and 5, anti-Plx1 antibodies; lanes 4 and 6, pre-immune IgG. The recovery of Plx1 in the immunoprecipitates was checked by immunoblotting with anti-Plx1 antibodies (lanes 7–9). Lanes 7 and 8, immunoprecipitates prepared with anti-Plx1 antibodies; lanes 9 and 10, immunoprecipitates prepared with pre-immune IgG. Molecular weight markers are indicated in kDa.

To compare the activity of Plx1 in M and I phase extracts, immunoprecipitations were performed and kinase activity assayed with casein as a substrate. Plx1 was 5- to 10-fold more active when isolated in its M phase form as compared with the I phase form (Figure 1, lanes 3 and 5). Comparable amounts of Plx1 protein were recovered from both extracts, and the isolated proteins displayed the characteristic mobility difference already observed in whole M and I phase extracts (Figure 1, lanes 7 and 8). No Plx1 protein and no casein kinase activity could be detected in control immunoprecipitates (Figure 1, lanes 4, 6, 9 and 10). These results indicate that in M phase-arrested extracts Plx1 is activated by a post-translational modification (presumably phosphorylation), confirming and extending recent data reported for *Drosophila* polo (Tavares *et al*., 1996) and Plk1 in somatic mammalian cells (Hamanaka *et al*., 1995; Mundt *et al*., 1997).

A dominant-negative approach suggests a role for Plx1 at M phase exit

To investigate the function of Plx1 in *Xenopus* egg extracts, we first chose a dominant-negative approach. Wild-type Plx1 (Plx1wt) and a catalytically inactive mutant [Plx1(N172A)], carrying a substitution of Asn172 by alanine, were purified to near homogeneity from recombinant baculovirus-infected Sf9 cells (Figure 2a). M phase extracts containing sperm nuclei were then supplemented with a 10-fold excess of Plx1wt or Plx1(N172A) (Figure 2b), and Ca^{2+} was added to trigger the metaphase to anaphase transition (Lohka and Maller, 1985; Lorca *et al*., 1993). In all extracts, both endogenous and recombinant Plx1 were stable throughout the experiment (Figure 2b). However, in those extracts that were supplemented with control buffer or Plx1wt, both endogenous Plx1 and recombinant Plx1wt displayed increased electrophoretic mobilities after a 90 min incubation (Figure 2b, compare lanes 2 and 4 with lanes 1 and 3, respectively), consistent with dephosphorylation of Plx1 upon exit from M phase

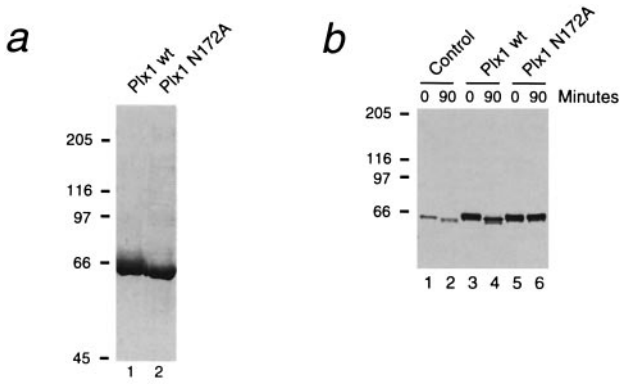

Fig. 2. Plx1(N172A), added in a 10-fold excess to M phase extracts, is not shifted to its interphase position upon Ca^{2+} addition. (**a**) Coomassie Blue staining of purified histidine-tagged Plx1wt (lane 1) and Plx1(N172A) (lane 2) resolved by SDS–PAGE (6 μg each). The gel was overloaded to estimate the quality of the purification. (**b**) Samples of M phase extracts supplemented with control buffer (lanes 1 and 2), Plx1wt (lanes 3 and 4) or Plx1(N172A) (lanes 5 and 6) from time points 0 (lanes 1, 3 and 5) and 90 min after Ca^{2+} addition (lanes 2, 4 and 6) were analysed for their content in Plx1 by immunoblotting. The slower migration of recombinant Plx1wt and Plx1 N172A, as compared with endogenous Plx1, is due to the presence of the N-terminal His6 tag. Note that exogenous Plx1 was added at ~10-fold excess over the endogenous kinase.

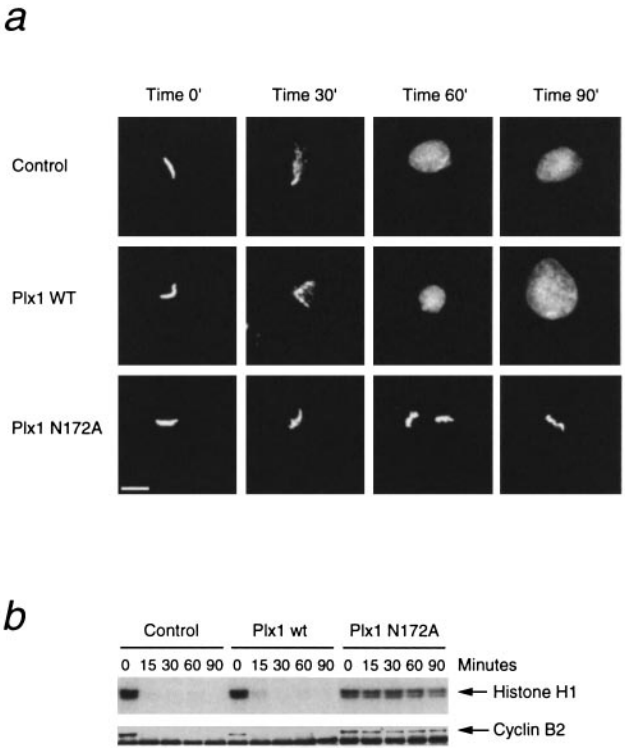

Fig. 3. Catalytically inactive Plx1 blocks the release of M phase-arrested *Xenopus* egg extracts into interphase. (**a**) M phase extracts containing sperm chromatin were supplemented with buffer (control, top panel), Plx1wt (middle panel) or Plx1(N172A) (lower panel). Samples were taken at the indicated times after Ca^{2+} addition (min), and chromatin was stained with Hoechst 33258. After 90 min, 95% of the input sperm DNA had decondensed in the control sample and 93% in the sample supplemented with Plx1 wt. In contrast, not a single example of decondensed chromatin could be found in the sample supplemented with Plx1(N172A). In each case, at least 300 sperm nuclei were counted. Scale bar denotes 5 μm. (**b**) Histone H1 kinase activity measurements (top panel), and cyclin B2 levels determined by immunoblotting (lower panel). The immunoreactive band migrating ahead of cyclin B2 represents an unidentified cross-reacting protein and provides an internal control for loading.

(see also Figure 1). Interestingly, no such mobility shift was observed in the extracts containing Plx1(N172A) (Figure 2b, lanes 5 and 6), suggesting that these extracts were blocked in M phase.

To visualize this putative M phase arrest more directly, cell cycle stages of the various extracts were monitored by microscopic inspection of added sperm chromatin (Figure 3a). After 60 min, control extracts as well as the extracts supplemented with Plx1wt had entered interphase, as indicated by the decondensation of sperm chromatin and the formation of nuclear envelopes (Figure 3a, top and middle rows). In striking contrast, when the Plx1(N172A) mutant was added, sperm chromatin remained condensed even after 90 min of incubation (Figure 3, bottom row), consistent with the view that the catalytically inactive Plx1 had caused a block to the exit from M phase. In parallel, we determined histone H1 kinase activity, a direct measure of MPF (Murray, 1991), and the stability of endogenous cyclin B2 in the very same extracts (Figure 3b). In both control extracts and extracts supplemented with Plx1wt, histone H1 kinase activity dropped drastically within 15 min after Ca^{2+} addition, but it remained high in the extract containing the catalytically inactive Plx1(N172A) (Figure 3b, top panel). Likewise, endogenous cyclin B2 was degraded rapidly in the former extracts, but was entirely stable in the presence of Plx1(N172A) (Figure 3b, lower panel).

Taken together, the results described above demonstrate that the presence of catalytically inactive Plx1 blocks the Ca^{2+}-induced release of *Xenopus* egg extracts from M phase arrest and prevents both cyclin destruction and MPF inactivation. The most likely interpretation of these results is that a 10-fold excess of mutant Plx1(N172A) protein prevents the M to I phase transition by exerting a dominant-negative effect over the endogenous wild-type Plx1, presumably by competing for Plx1 substrates or other interacting proteins.

Immunodepletion–reconstitution experiments confirm a requirement for Plx1 activity during M phase exit

To corroborate and extend the above findings, immunodepletion and reconstitution experiments were performed. M phase extracts were treated with protein A beads that had been coated with either anti-Plx1 antibodies or pre-immune IgG as a control. More than 95% of Plx1 could be depleted by anti-Plx1 antibody beads (Figure 4a, lane 3), whereas control depletion did not significantly alter the level of Plx1 in the extracts (Figure 4a, lane 2). Also, overall protein profiles were unaffected by both treatments, as determined by Coomassie blue staining (data not shown). The depleted extracts were then tested for their ability to undergo the M to I phase transition in response to Ca^{2+}. Whereas >95% of the sperm nuclei in the control depleted extracts underwent chromatin decondensation and nuclear assembly, the extracts lacking Plx1 could not be released from the metaphase block upon Ca^{2+} addition and virtually all sperm chromatin remained condensed (Figure 4b, first and second rows). The addition of a roughly physiological amount of recombinant Plx1wt protein (150 nM final concentration; see Figure 4a, lane 4)

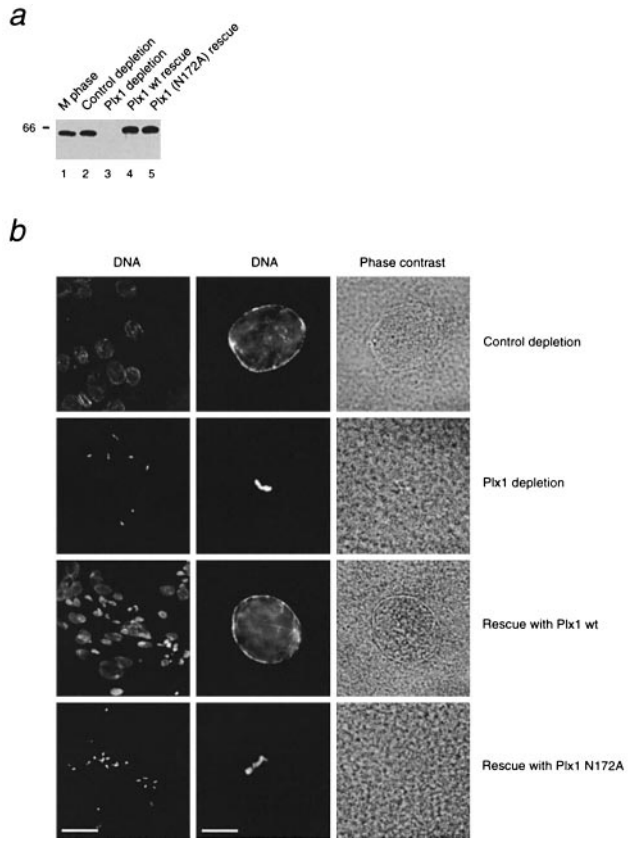

Fig. 4. A reversible block to M phase exit by immunodepletion–reconstitution of Plx1. Fresh M phase extracts were depleted of Plx1 and assayed for their ability to be released into interphase by Ca^{2+} addition. Furthermore, the ability of Plx1wt or Plx1(N172A) to rescue the depletion phenotype was tested. (**a**) Immunoblot showing Plx1 levels in untreated M phase extracts (lane 1), in extracts treated with either control pre-immune beads (lane 2) or anti-Plx1 beads (lane 3), and in Plx1-depleted extracts supplemented with recombinant Plx1wt (lane 4) or Plx1(N172A) (lane 5). Note that recombinant Plx1wt and Plx1(N172A) display a slightly retarded migration, as compared with endogenous Plx1, due to the presence of an N-terminal His6 tag. Molecular weight markers are indicated in kDa. (**b**) Depleted extracts were supplemented with sperm chromatin and either control buffer, Plx1wt or Plx1(N172A), as indicated. The assembly of nuclei was monitored 120 min after Ca^{2+} addition by staining chromatin with Hoechst 33258 (left panels, two magnifications are shown) and by phase contrast microscopy (right panels). The scale bars in the left and central panels denote 20 and 5 μm, respectively.

restored the ability of the Plx1-depleted extracts to exit M phase (Figure 4b, third row), but the addition of the Plx1(N172A) mutant (Figure 4a, lane 5) did not (Figure 4b, fourth row). In a typical rescue experiment, 75–85% of the sperm nuclei decondensed in response to Plx1wt. All extracts remained arrested in M phase in the absence of Ca^{2+} addition, indicating that immunodepletion had not caused spontaneous release from the metaphase block (data not shown). These results confirm that Plx1 activity is required for the Ca^{2+}-induced release of CSF-arrested *Xenopus* egg extracts from M phase arrest.

Plx1 activity is required for the proteolytic degradation of multiple mitotic regulators

Progression through mitosis requires the ubiquitin-dependent proteolytic degradation of multiple proteins (Deshaies, 1995; King *et al*., 1996). The data shown above clearly demonstrate that a catalytically inactive Plx1 blocks the degradation of a cyclin, but they do not distinguish between a generalized block affecting the entire degradation machinery from a block exerted at the level of a specific substrate. We considered it important, therefore, to determine whether the inability of extracts supplemented with Plx1(N172A) to destroy the mitotic cyclins could be extended to a protein that is expected to be degraded at the metaphase to anaphase transition. Although vertebrate homologues of Pds1p/Cut2 have not yet been identified, the destruction of both Pds1p and Cut2 appears to be faithfully reproduced in *Xenopus* extracts (Cohen-Fix *et al*., 1996; Funabiki *et al*., 1997). We thus performed cell-free destruction assays (Lohka and Maller, 1985; Murray *et al*., 1989; Stewart *et al*., 1994), using M phase extracts containing either Plx1wt, Plx1(N172A) or control buffer, supplemented with *in vitro* translated substrates for APC-dependent proteolytic destruction.

When extracts were supplemented with a mixture of *in vitro* translated full-length cyclin B2 and a non-destructible cyclin B2 mutant (cyclin B2ΔN7–69) as an internal control, the full-length cyclin B2 was destroyed upon Ca^{2+} addition both in control extracts and in extracts supplemented with Plx1wt (Figure 5a, top and middle panels). However, cyclin B2 was entirely stable in the presence of Plx1(N172A) (Figure 5a, bottom panel), confirming and extending the results shown above (Figure 3b). The non-destructible cyclin B2 mutant was stable in all extracts, as expected. The fate of Cut2 was then investigated following the same procedure. Like cyclin B2, Cut2 was partially destroyed upon Ca^{2+} addition both in control extracts and in extracts supplemented with Plx1wt (Figure 5b, top and middle panels), but was entirely stable in the presence of Plx1(N172A) (Figure 5b, bottom panel). In agreement with previous results (Funabiki *et al*., 1996, 1997), only ~50% of Cut2 was destroyed in these assays (confirmed by densitometric scanning; not shown). Moreover, the Cut2 protein migrated as multiple isoforms, reflecting extensive phosphorylation in M phase extracts (Funabiki *et al*., 1997). While these isoforms could be seen readily in all extracts at time 0, the non-degraded Cut2 protein remaining in control extracts and in extracts supplemented with Plx1wt (Figure 5b, top and middle panels, time 90 min, plus Ca^{2+}) was clearly shifted to lower migrating forms, probably due to dephosphorylation upon exit of these extracts from M phase. No degradation and no alterations in the migration of Cut2 could be observed in the presence of Plx1(N172A), indicating that the corresponding extracts were unable to degrade a substrate which, at least in yeast, is known to be degraded before the onset of anaphase. Regardless of whether or not *S.pombe* Cut2 mimics the fate of its (hypothetical) *Xenopus* homologue, these results clearly indicate that the block to proteolysis imposed by the addition of the Plx1(N172A) mutant to *Xenopus* egg extracts is not limited to a particular substrate.

Plx1 activity is required for the degradation of c-Mos

In *Xenopus* egg extracts, the arrest in meiotic metaphase II is imposed by an activity known as cytostatic factor (CSF), which comprises a MAP kinase cascade under the control of the proto-oncogene product c-Mos (Sagata *et al*., 1989) (reviewed in Sagata, 1997). In response to

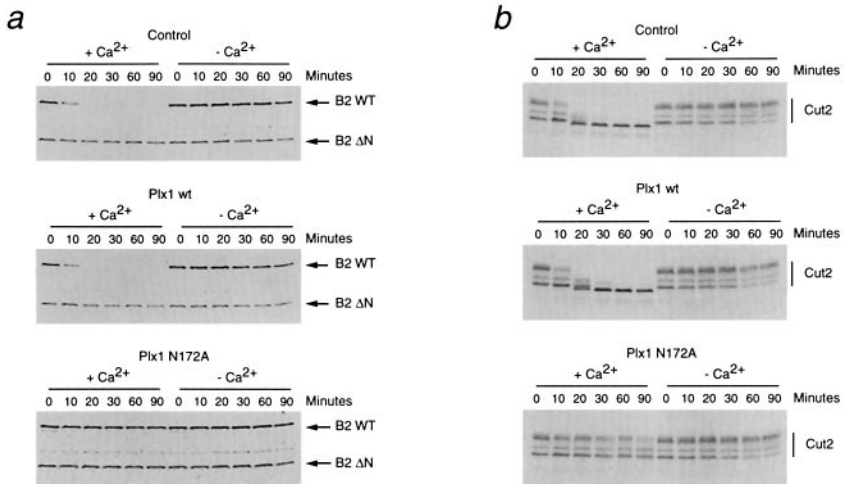

Fig. 5. The destruction of both mitotic cyclins and Cut2 requires Plx1 activity. Destruction assays were performed in M phase extracts supplemented with control buffer (top panels), Plx1wt (middle panels) or Plx1(N172A) (lower panels), and a mixture of *in vitro* translated full-length cyclin B2 and cyclin B2ΔN7–69 (**a**) or *S.pombe* Cut2 (**b**) as substrates for degradation. The positions of full-length cyclin B2 (B2 wt) and cyclin B2ΔN7–69 (B2 ΔN) (a), and Cut2 (b), as detected by autoradiography, are indicated. Note the complex migration pattern of Cut2 polypeptides, which is due to extensive phosphorylation in M phase extracts.

Ca^{2+}, the proteolytic degradation system is activated in *Xenopus* egg extracts, before the inhibitory MAP kinase pathway is inactivated through degradation of c-Mos (Ferrell *et al*., 1991; Lorca *et al*., 1991; Watanabe *et al*., 1991). Since c-Mos itself is degraded in a ubiquitin-dependent manner by the 26S proteasome (Ishida *et al*., 1993), it was of interest to determine the fate of c-Mos in an extract in which the activation of the proteolytic machinery was blocked by Plx1(N172A). As shown by immunoblotting, Ca^{2+}-dependent degradation of c-Mos could be visualized readily in extracts supplemented with control buffer or Plx1wt (Figure 6, left and center panels, lanes 2). In contrast, c-Mos was entirely stable in the extract supplemented with the Plx1(N172A) mutant (Figure 6, right panel, lane 2). Also, c-Mos was stable in all extracts in the absence of Ca^{2+} (Figure 6, lanes 3 and 4). Together with the results described in the previous sections, these data demonstrate that Plx1(N172A) prevents the degradation of all substrates of ubiquitin-dependent proteolysis tested. The most straightforward interpretation of these results is that Plx1 activity is required for the functional activation of the APC-dependent proteolytic machinery during M phase exit.

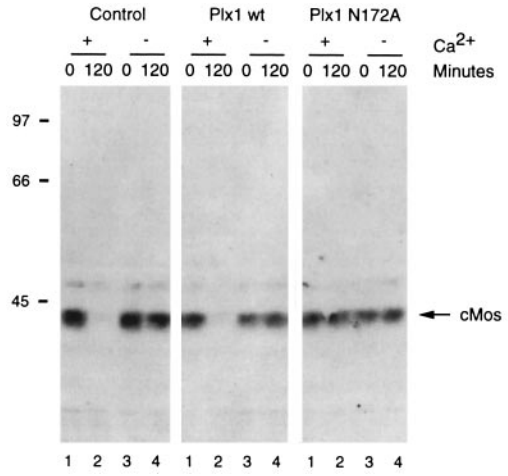

Fig. 6. c-Mos degradation is prevented by Plx1(N172A). Fresh M phase extracts were supplemented with buffer (control, left panel), Plx1wt (middle panel) or Plx1(N172A) (right panel). Samples were taken at the indicated times after Ca^{2+} addition (min), and their content in c-Mos analysed by immunoblotting. The position of c-Mos (arrow) as well as the molecular weight markers (in kDa) are indicated.

Discussion

This study reveals a novel role for Plx1 in cell cycle progression. Both a dominant-negative approach and immunodepletion–reconstitution experiments concur to indicate that Plx1 activity is required for the activation of the proteolytic degradation machinery in *Xenopus* egg extracts. In the absence of Plx1 activity, the Ca^{2+}-dependent activation of the APC-mediated degradation pathway is blocked, with the result that all mitotic targets analysed, i.e. mitotic cyclins, *S.pombe* Cut2 and c-Mos, remain stable. As a consequence, extracts remain arrested in M phase with high MPF activity.

Xenopus eggs are arrested in meiotic metaphase II, because CSF, a c-Mos-dependent MAP kinase cascade (Sagata, 1997), prevents the APC-dependent degradation pathway from being turned on (Abrieu *et al*., 1996). Upon fertilization, a rise in Ca^{2+} triggers the release from this block. Our data identify Plx1 as an important new element in the functional activation of the APC-dependent degradation pathway. Formally, active Plx1 counteracts the block imposed by the c-Mos/MAP kinase pathway. However, when interpreting our results, it is important to bear in mind that the functional activation of the proteolytic machinery precedes the inactivation of the c-Mos/MAP kinase pathway (Ferrell *et al*., 1991; Lorca *et al*., 1991; Watanabe *et al*., 1991). In other words, the inactivation of the c-Mos/MAP kinase pathway is the consequence rather than the cause of the Ca^{2+}-induced release from the metaphase II block. Thus, active Plx1 almost certainly acts downstream of the c-Mos/MAP kinase pathway.

Figure 7 summarizes, in highly schematic form, a

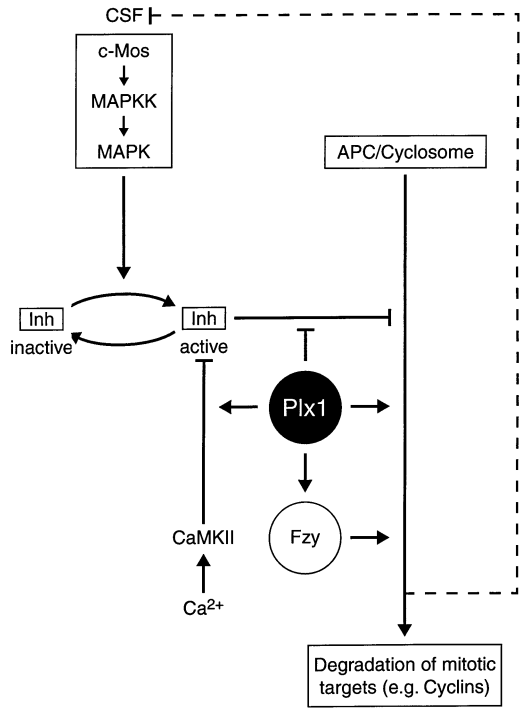

Fig. 7. Plx1 as a regulator of the M phase degradation machinery in *Xenopus* egg extracts. M phase extracts are arrested in meiotic metaphase II, due to the action of the c-Mos/MAP kinase pathway (CSF). According to the model presented here, the c-Mos/MAP kinase pathway promotes the activation of an inhibitor of APC/cyclosome function. In response to Ca^{2+}, APC is relieved from the action of this inhibitor, allowing it to degrade its substrates. Subsequently, c-Mos is degraded and the MAP kinase pathway is turned off. As depicted, Plx1 is proposed to (i) mediate the Ca^{2+}-dependent inactivation of the hypothetical APC inhibitor, (ii) counteract the effect of this inhibitor, (iii) maintain the activity of APC, or (iv) contribute to the functional coupling between APC and co-factors of the Fzy family. These mechanisms are not mutually exclusive, and other modes of action cannot be excluded. In particular, it would be premature to exclude regulation of the degradation machinery at the level of substrate presentation to the proteasome.

tentative model for how APC-mediated degradation might be regulated in *Xenopus* egg extracts. This model also illustrates possible sites of action for Plx1. It might seem straightforward to argue that Plx1 could directly phosphorylate subunits of APC and thereby cause the activation of the complex, and/or be important for maintaining its activity. However, the situation may be more complicated, as our data clearly indicate a requirement for Plx1 activity after the Ca^{2+} signal. In fact, Plx1 is active already during M phase arrest (see Figure 1) and so is APC, at least when assayed *in vitro* after immunoprecipitation from M phase extracts (King *et al*., 1995; Peters *et al*., 1996). To reconcile these findings with the fact that none of the targets of APC are degraded during M phase arrest, it is tempting to postulate the existence of an inhibitor of APC (see Figure 7, and Minshull *et al*., 1994). This hypothetical APC inhibitor would be maintained in an active state by the c-Mos/MAP kinase pathway (CSF), but would be inactivated in response to a Ca^{2+} signal. According to this scheme, Plx1 activity might be required for inactivation of the inhibitor or for neutralizing its inhibitory action.

Alternatively, it is possible that Plx1 might function in concert with proteins of the Fzy family (Figure 7). Two *Drosophila* genes, *fizzy (fzy)* and *fizzy-related (fzr)*, recently were shown to be required for the degradation of mitotic cyclins during mitosis and G_1 phase of the cell cycle, respectively (Sigrist *et al*., 1995; Sigrist and Lehner, 1997). Similarly, putative homologues of *fzy* and *fzr* have been identified in *S.cerevisiae*: whereas Cdc20p, a potential homologue of *fzy*, is essential for the degradation of Pds1p at anaphase onset, Hct1p/Cdh1p, a homologue of *fzr*, is required for the destruction of Clb2 and for exit from mitosis (Schwab *et al*., 1997; Visintin *et al*., 1997; Shirayama *et al*., 1998). Both the *Drosophila* and the yeast proteins contain WD40 repeats likely to mediate protein–protein interactions, but their precise biochemical functions remain unknown. In support of a functional relationship between Plks and Fzy/Cdc20p-like proteins, the M phase block imposed by catalytically inactive Plx1 in *Xenopus* egg extracts is reminiscent of the mitotic arrest caused by mutational inactivation of the *fzy* gene in *Drosophila* (Sigrist *et al*., 1995). Thus, it might be rewarding to test members of the Fzy family as potential substrates of Plks.

To what extent Plk activity is required for the destruction of APC targets in somatic cells remains to be determined. However, it is clear already that a requirement for Plks in M phase proteolysis is not limited to *Xenopus* egg extracts. Recent studies performed in *S.cerevisiae* in fact indicate that Cdc5p function is necessary for the degradation of mitotic cyclins during mitosis (Shirayama *et al*., 1998). One interesting difference between the results reported here and those obtained in budding yeast concerns the fate of APC targets that are degraded prior to anaphase onset. While degradation of *S.pombe* Cut2 was blocked in the *Xenopus* egg extracts supplemented with catalytically inactive Plx1, Pds1p was degraded in the absence of *CDC5* function in *S.cerevisiae* (Shirayama *et al*., 1998). Possible explanations for this discrepancy may relate to the absence of G_1 and G_2 phases in the rapid early embryonic cell cycles of *Xenopus*, and/or to the particular temporal choreography of spindle formation, sister chromatid separation and cytokinesis in budding yeast.

In somatic cells, the activity of APC is thought to be controlled by checkpoints that monitor the integrity of the spindle apparatus and the bipolar attachment of chromosomes to microtubules (Rudner and Murray, 1996). In view of the implication of a MAP kinase cascade in such checkpoint pathways (Minshull *et al*., 1994; Takenaka *et al*., 1997), and the formal antagonism between Plx1 and the c-Mos/MAP kinase (CSF) pathway described here, it will be interesting to explore a possible function for Plks in releasing APC from checkpoint-induced M phase blocks.

In conclusion, our findings strengthen the emerging view that Plks may contribute to regulate the activity of Cdk–cyclin complexes at multiple stages of mitotic progression. In general terms, we propose that Plks are important not only for bipolar spindle assembly and the activation of Cdk–cyclin complexes at the onset of mitosis, but are also required for the destruction of APC targets upon exit from M phase.

Materials and methods

Extract preparation and manipulations

CSF-arrested egg extracts (M phase) and activated egg extracts (I phase) were prepared as described (Murray, 1991), except that egg activation

was performed with Ca^{2+} ionophore A 23187 (Sigma) for 5 min and Versilube oil was omitted. Demembranated sperm nuclei were obtained as described (Murray, 1991). For the dominant-negative approach, extracts were supplemented with sperm nuclei (200 nuclei/μl) and 10% (v/v) of a solution containing recombinant Plx1 proteins (1 mg/ml) as indicated, and $CaCl_2$ was added to 0.4 mM final concentration. Similar results were obtained using Plx1 purified from Sf9 cells with or without prior activation by okadaic acid treatment (Kumagai and Dunphy, 1996). For immunodepletion experiments, protein A beads (Affiprep, Bio-Rad) were pre-coated with anti-Plx1 antibodies (see below) or IgG purified from pre-immune serum (10 mg IgG/ml of beads) and washed in CSF-XB buffer (Murray, 1991). Beads were added to M phase extracts (20% v/v) containing 250 μg/ml cyclohexamide, and samples were incubated for 1 h at 4°C with rotation. Beads were removed by centrifugation, and the supernatants supplemented with sperm nuclei (200 nuclei/μl). Then, recombinant Plx1 proteins isolated from okadaic acid-treated Sf9 cells (Kumagai and Dunphy, 1996) were added as indicated, and $CaCl_2$ was added to 0.4 mM final concentration. Samples were processed for microscopic inspection as described (Murray, 1991). Pictures were taken using a Zeiss Axioplan 2 microscope equipped with a CCD camera (Quantix, Photometrics), and processed with IP LabSpectrum and Adobe Photoshop software.

Preparation of recombinant Plx1

N-terminally His6-tagged Plx1wt and Plx1(N172A) were purified from baculovirus-infected Sf9 cells as described (Kumagai and Dunphy, 1996), except that proteins were eluted from the Ni^{2+} NTA agarose beads (Qiagen) with EB buffer [10 mM HEPES (pH 7.7), 25 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 50 mM sucrose, 200 mM imidazole, 10 μg/ml each of leupeptin, pepstatin and chymostatin, and 5 mM β-mercaptoethanol]. For reconstitution of depleted extracts, Plx1wt and N172A were diluted in 10 mM HEPES (pH 7.7), 10 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 50 mM sucrose, 10 μg/ml each of leupeptin, pepstatin and chymostatin, and 5 mM β-mercaptoethanol.

Immunoblotting, immunoprecipitations and kinases assays

His6-tagged Plx1wt was isolated as described above, gel purified and used to raise antibodies in rabbits. Immunoblotting experiments were performed on 0.5 μl samples of extracts (25 μg protein) resolved by SDS–PAGE, using anti-Plx1 polyclonal serum (1/5000 dilution), anti-cyclin B2 monoclonal antibodies (10 μg/ml) or anti-c-mos polyclonal serum (1/2000 dilution). For Plx1 immunoprecipitations, protein A beads (Affiprep, Bio-Rad) were pre-coated with anti-Plx1 antibodies or pre-immune serum, and added to 10 μl of extracts that had been diluted 10-fold in ES buffer [80 mM β-glycerophosphate, 20 mM EGTA, 50 mM NaF, 0.1% CHAPS, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml each of leupeptin, pepstatin and chymostatin]. After recovery by centrifugation, beads were washed twice with ES buffer and twice with kinase buffer [20 mM HEPES (pH 7.7), 15 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM NaF]. Casein kinase assays were performed in kinase buffer containing 1 mM dithiothreitol (DTT), 100 μM ATP, 1 μCi [γ-^{32}P]ATP and 100 μg/ml casein at 23°C for 30 min. Histone H1 kinase assays were done as described (Murray, 1991), except that NP-40 was omitted from the buffer, and reactions were carried out at 23°C for 10 min.

Destruction assays

Substrates were prepared by coupled *in vitro* transcription–translation in reticulocyte lysates (TNT, Promega), in the presence of [^{35}S]cysteine/methionine (Amersham), using the plasmids pCMVcycB2 (Gallant and Nigg, 1992) (B2 wt), pCMVcycB2ΔN7–69 (B2ΔN7–69) (G.Maridor and E.A.Nigg, unpublished results) or pHY22 (Cut2) (Funabiki *et al*., 1997). M phase extracts were supplemented with control buffer, Plx1wt or Plx1(N172A) as described above, and with lysates containing the substrates (10% v/v). Then, $CaCl_2$ was added to 0.4 mM final concentration where indicated (Stewart *et al*., 1994). Samples taken at the indicated times were resolved by SDS–PAGE and proteins detected by autoradiography.

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References

Abrieu,A., Lorca,T., Labbe,J.C., Morin,N., Keyse,S. and Doree,M. (1996) MAP kinase does not inactivate, but rather prevents the cyclin degradation pathway from being turned on in *Xenopus* egg extracts. *J. Cell Sci.*, **109**, 239–246.

Cohen-Fix,O., Peters,J.M., Kirschner,M.W. and Koshland,D. (1996) Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.*, **10**, 3081–3093.

Deshaies,R.J. (1995) The self-destructive personality of a cell cycle in transition. *Curr. Opin. Cell Biol.*, **7**, 781–789.

Ferrell,J.E.,Jr, Wu,M., Gerhart,J.C. and Martin,G.S. (1991) Cell cycle tyrosine phosphorylation of p34cdc2 and a microtubule-associated protein kinase homolog in *Xenopus* oocytes and eggs. *Mol. Cell. Biol.*, **11**, 1965–1971.

Funabiki,H., Yamano,H., Kumada,K., Nagao,K., Hunt,T. and Yanagida,M. (1996) Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature*, **381**, 438–441.

Funabiki,H., Yamano,H., Nagao,K., Tanaka,H., Yasuda,H., Hunt,T. and Yanagida,M. (1997) Fission yeast Cut2 required for anaphase has two destruction boxes. *EMBO J.*, **16**, 5977–5987.

Gallant,P. and Nigg,E.A. (1992) Cyclin B2 undergoes cell cycle-dependent nuclear translocation and, when expressed as a non-destructible mutant, causes mitotic arrest in HeLa cells. *J. Cell Biol.*, **117**, 213–224.

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

Glover,D.M., Ohkura,H. and Tavares,A. (1996) Polo kinase: the choreographer of the mitotic stage? *J. Cell Biol.*, **135**, 1681–1684.

Golsteyn,R.M., Mundt,K.E., Fry,A.M. and Nigg,E.A. (1995) Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle function. *J. Cell Biol.*, **129**, 1617–1628.

Grieco,D., Porcellini,A., Avvedimento,E.V. and Gottesman,M.E. (1996) Requirement for cAMP-PKA pathway activation by M phase-promoting factor in the transition from mitosis to interphase. *Science*, **271**, 1718–1723.

Hamanaka,R., Smith,M.R., O'Connor,P.M., Maloid,S., Mihalic,K., Spivak,J.L., Longo,D.L. and Ferris,D.K. (1995) Polo-like kinase is a cell cycle-regulated kinase activated during mitosis. *J. Biol. Chem.*, **270**, 21086–21091.

Hardy,C.F. and Pautz,A. (1996) A novel role for Cdc5p in DNA replication. *Mol. Cell. Biol.*, **16**, 6775–6782.

Hartley,R.S., Rempel,R.E. and Maller,J.L. (1996) *In vivo* regulation of the early embryonic cell cycle in *Xenopus*. *Dev. Biol*, **173**, 408–419.

Hartwell,L.H., Mortimer,R.K., Culotti,J. and Culotti,M. (1973) Genetic control of the cell division cycle in yeast: V. Genetic analysis of cdc mutants. *Genetics*, **74**, 267–286.

Hershko,A. and Ciechanover,A. (1992) The ubiquitin system for protein degradation. *Annu. Rev. Biochem.*, **61**, 761–807.

Holloway,S.L., Glotzer,M., King,R.W. and Murray,A.W. (1993) Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell*, **73**, 1393–1402.

Hunt,T., Luca,F.C. and Ruderman,J.V. (1992) The requirements for protein synthesis and degradation, and the control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo. *J. Cell Biol.*, **116**, 707–724.

Irniger,S., Piatti,S., Michaelis,C. and Nasmyth,K. (1995) Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell*, **81**, 269–278.

Ishida,N., Tanaka,K., Tamura,T., Nishizawa,M., Okazaki,K., Sagata,N. and Ichihara,A. (1993) Mos is degraded by the 26S proteasome in a ubiquitin-dependent fashion. *FEBS Lett.*, **324**, 345–348.

Jentsch,S. and Schlenker,S. (1995) Selective protein degradation: a journey's end within the proteasome. *Cell*, **82**, 881–884.

King,R.W., Peters,J.M., Tugendreich,S., Rolfe,M., Hieter,P. and Kirschner,M.W. (1995) A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell*, **81**, 279–288.

King,R.W., Deshaies,R.J., Peters,J.M. and Kirschner,M.W. (1996) How proteolysis drives the cell cycle. *Science*, **274**, 1652–1659.

Kitada,K., Johnson,A.L., Johnston,L.H. and Sugino,A. (1993) A multicopy suppressor gene of the *Saccharomyces cerevisiae* G_1 cell cycle mutant gene *dbf4* encodes a protein kinase and is identified as *CDC5*. *Mol. Cell. Biol.*, **13**, 4445–4457.

Kumagai,A. and Dunphy,W.G. (1996) Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science*, **273**, 1377–1380.

Lahav-Baratz,S., Sudakin,V., Ruderman,J.V. and Hershko,A. (1995) Reversible phosphorylation controls the activity of cyclosome-associated cyclin-ubiquitin ligase. *Proc. Natl Acad. Sci. USA*, **92**, 9303–9307.

Lane,H.A. and Nigg,E.A. (1996) Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J. Cell Biol.*, **135**, 1701–1713.

Lane,H.A. and Nigg,E.A. (1997) Cell-cycle control: POLO-like kinases join the outer circle. *Trends Cell Biol.*, **7**, 63–68.

Lohka,M.J. and Maller,J.L. (1985) Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. *J. Cell Biol.*, **101**, 518–523.

Lorca,T., Galas,S., Fesquet,D., Devault,A., Cavadore,J.C. and Doree,M. (1991) Degradation of the proto-oncogene product p39mos is not necessary for cyclin proteolysis and exit from meiotic metaphase: requirement for a Ca(2+)-calmodulin dependent event. *EMBO J.*, **10**, 2087–2093.

Lorca,T., Cruzalegui,F.H., Fesquet,D., Cavadore,J.C., Mery,J., Means,A. and Doree,M. (1993) Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature*, **366**, 270–273.

Minshull,J., Sun,H., Tonks,N.K. and Murray,A.W. (1994) A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. *Cell*, **79**, 475–486.

Mundt,K.E., Golsteyn,R.M., Lane,H.A. and Nigg,E.A. (1997) On the regulation and function of human polo-like kinase 1 (PLK1): effects of overexpression on cell cycle progression. *Biochem. Biophys. Res. Commun.*, **239**, 377–385.

Murray,A.W. (1991) Cell cycle extracts. *Methods Cell Biol.*, **36**, 581–605.

Murray,A.W., Solomon,M.J. and Kirschner,M.W. (1989) The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature*, **339**, 280–286.

Nigg,E.A. (1995) Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *BioEssays*, **17**, 471–480.

Ohkura,H., Hagan,I.M. and Glover,D.M. (1995) The conserved *Schizosaccharomyces pombe* kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G_1 and G_2 cells. *Genes Dev.*, **9**, 1059–1073.

Peters,J.M., King,R.W., Hoog,C. and Kirschner,M.W. (1996) Identification of BIME as a subunit of the anaphase-promoting complex. *Science*, **274**, 1199–1201.

Rempel,R.E., Sleight,S.B. and Maller,J.L. (1995) Maternal *Xenopus* Cdk2–cyclin E complexes function during meiotic and early embryonic cell cycles that lack a G1 phase. *J. Biol. Chem.*, **270**, 6843–6855.

Rudner,A.D. and Murray,A.W. (1996) The spindle assembly checkpoint. *Curr. Opin. Cell Biol.*, **8**, 773–780.

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

Sagata,N., Watanabe,N., Vande Woude,G.F. and Ikawa,Y. (1989) The c-*mos* proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature*, **342**, 512–518.

Schwab,M., Lutum,A.S. and Seufert,W. (1997) Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell*, **90**, 683–693.

Shirayama,M., Zachariae,W., Ciosk,R. and Nasmyth,K. (1998) The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J.*, **17**, 1336–1349.

Sigrist,S.J. and Lehner,C.F. (1997) *Drosophila fizzy-related* down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell*, **90**, 671–681.

Sigrist,S., Jacobs,H., Stratmann,R. and Lehner,C.F. (1995) Exit from mitosis is regulated by *Drosophila fizzy* and the sequential destruction of cyclins A, B and B3. *EMBO J.*, **14**, 4827–4838.

Stewart,E., Kobayashi,H., Harrison,D. and Hunt,T. (1994) Destruction of *Xenopus* cyclins A and B2, but not B1, requires binding to p34cdc2. *EMBO J.*, **13**, 584–594.

Sudakin,V., Ganoth,D., Dahan,A., Heller,H., Hershko,J., Luca,F.C., Ruderman,J.V. and Hershko,A. (1995) The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Mol. Biol. Cell*, **6**, 185–197.

Sunkel,C.E. and Glover,D.M. (1988) *polo*, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J. Cell Sci.*, **89**, 25–38.

Surana,U., Amon,A., Dowzer,C., McGrew,J., Byers,B. and Nasmyth,K. (1993) Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.*, **12**, 1969 1978.

Takenaka,K., Gotoh,Y. and Nishida,E. (1997) MAP kinase is required for the spindle assembly checkpoint but is dispensable for the normal M phase entry and exit in *Xenopus* egg cell cycle extracts. *J. Cell Biol.*, **136**, 1091–1097.

Tavares,A.A., Glover,D.M. and Sunkel,C.E. (1996) The conserved mitotic kinase polo is regulated by phosphorylation and has preferred microtubule-associated substrates in *Drosophila* embryo extracts. *EMBO J.*, **15**, 4873–4883.

Toczyski,D.P., Galgoczy,D.J. and Hartwell,L.H. (1997) CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. *Cell*, **90**, 1097–1106.

Varshavsky,A. (1997) The ubiquitin system. *Trends Biochem. Sci.*, **22**, 383–387.

Visintin,R., Prinz,S. and Amon,A. (1997) CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science*, **278**, 460–463.

Watanabe,N., Hunt,T., Ikawa,Y. and Sagata,N. (1991) Independent inactivation of MPF and cytostatic factor (Mos) upon fertilization of *Xenopus* eggs. *Nature*, **352**, 247–248.

Yamada,H., Kumada,K. and Yanagida,M. (1997) Distinct subunit functions and cell cycle regulated phosphorylation of 20S APC/cyclosome required for anaphase in fission yeast. *J. Cell Sci.*, **110**, 1793–1804.

Zachariae,W., Shin,T.H., Galova,M., Obermaier,B. and Nasmyth,K. (1996) Identification of subunits of the anaphase-promoting complex of *Saccharomyces cerevisiae*. *Science*, **274**, 1201–1204.

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