Physical and functional interactions between polo kinase and the spindle pole component Cut12 regulate mitotic commitment in *S. pombe*

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Commitment to mitosis is regulated by a protein kinase complex called MPF. MPF is inhibited by Wee1-related kinases and activated by Cdc25 phosphatase. MPF activation further boosts Cdc25 and represses Wee1. This feedback control probably involves polo kinase. A dominant *cut12.s11* mutation in the *Schizosaccharomyces pombe* spindle pole body (SPB) component Cut12 both suppresses the conditional lethal mitotic commitment defect of *cdc25.22* and promotes premature association of the *S. pombe* polo kinase, Plo1, with the SPB. We now show that Cut12 associated with Plo1 in two hybrid and immunoprecipitation assays. Plo1 function was required for recognition of the mitotic SPB by the phospho-specific antibody MPM-2. In vivo MPM-2 staining and in vitro kinase assays established that the loss-of-function mutation, *cut12.1*, reduced mitotic activation of Plo1, whereas the gain-of-function mutation, *cut12.s11*, promoted higher levels of Plo1 activity than were normally seen in interphase. *cut12.s11* could not promote mitotic commitment of *cdc25.22* cells when Plo1 function was compromised. Expression of a constitutively active *plo1* allele suppressed the mitotic commitment defect of *cdc25.22*. These data suggest that *cut12.s11* suppresses *cdc25.22* by promoting Plo1 activity. Furthermore, the delayed mitotic commitment of *plo1.ts2* cells suggests that Plo1 is an integral part of the core controls that modulate MPF activation in *S. pombe*.

Commitment to mitosis is regulated by the activity of a conserved protein kinase complex called MPF (Nurse 1990). Phosphorylation of MPF by protein kinases related to the fission yeast protein kinase Wee1 inhibit its activity. Removal of these phosphates by Cdc25 phosphatase activates MPF and triggers an autocatalytic feedback loop that represses Wee1 and further boosts Cdc25 activity (Hoffmann et al. 1993). Thus, the activation of the bulk of Cdc25 is actually dependent on MPF activity (Kovelman and Russell 1996). Feedback loop activation of Cdc25 involves phosphorylation by MPF itself and one or more additional kinases (Strausfeld et al. 1992; Izumi and Maller 1993, 1995). Polo kinase is a prime candidate for such a Cdc25-activating kinase. It is required for commitment to mitosis in nontransformed human cells and was purified from *Xenopus* egg extracts as a molecule that bound to, phosphorylated, and activated Cdc25 (Kumagai and Dunphy 1996; Lane and Nigg 1996). The phosphorylation of Cdc25 by *Xenopus* polo kinase (Plx1) conferred reactivity to the phospho-specific antibody MPM-2. This reactivity usually occurs on Cdc25 during mitotic commitment (Kuang et al. 1994; Kumagai and Dunphy 1996). Subsequent studies established that depletion of Plx1 from *Xenopus* oocytes delays mitotic commitment in a way that can be overcome by addition of excess Cdc25C (Qian et al. 1998). Plx1 activity is not required in *Xenopus* oocyte extracts for MPF activation per se; rather, its presence ensures the abrupt nonlinear kinetics of MPF activation (Karaiskou et al. 1999).

Although less is known about the regulation of Wee1 and the related kinase Myt1, it has been established that both become hyperphosphorylated upon mitotic commitment and that hyperphosphorylation of Wee1 correlated with a reduction in its kinase activity toward p34^{cdc2}/cyclin B (Tang et al. 1993; Honda et al. 1995; Mueller et al. 1995). Polo kinase may inhibit Wee1 during the feedback loop as the budding yeast polo kinase, Cdc5, binds to and phosphorylates the Wee1 kinase Swe1 (Bartholomew et al. 2001).

Studies in fission yeast have suggested that the spindle

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pole may play a critical role in regulating the commitment to mitosis. The *cut12+* gene encodes an essential protein that is required for spindle formation and associates with the cytoplasmic domain of the spindle pole body (SPB; Bridge et al. 1998). Dominant *cut12* mutations were isolated in a screen for suppressors of cdc twenty-five (stf). These *stf* mutants, such as *cut12.s11*, suppress the conditional lethality of *cdc25.22* and permit colony formation of *cdc25Δ* (Hudson et al. 1990). The fission yeast polo kinase, Plo1, normally displays strong association with the SPB of mitotic but not interphase cells (Mulvihill et al. 1999). In *cut12.s11*, strong SPB association of Plo1 is seen throughout the cell cycle (Mulvihill et al. 1999; Grallert and Hagan 2002). Thus, the dominant mutation in this SPB component simultaneously induces mitotic behavior of Plo1 and suppresses the *cdc25.22* mutation. Both processes are dependent on the activity of the NIMA-related kinase Fin1 (Grallert and Hagan 2002).

We now show that Plo1 binds to Cut12 and that Plo1 function is necessary for the *cut12.s11* mutation to promote the transition of the G2/M boundary when Cdc25 function is compromised. A key role for Plo1 in regulating mitotic commitment is supported by the observations that both MPF activation and mitotic commitment are delayed in the *plo1.ts2* loss-of-function mutant and that induction of the "constitutively active" *plo1.S124DT197D* mutant allele enables *cdc25.22* cells to divide under restrictive conditions. The loss-of-function mutant *cut12.1* shows that Cut12 is required for the full activation of Plo1 during mitotic commitment, whereas the gain-of-function mutation *cut12.s11* promotes abnormally high levels of Plo1 activity in interphase cells that lack Cdc2 activity. Because Plo1 activation is normally dependent on prior activation of MPF (Tanaka et al. 2001), we discuss these results in the context of a role for the localized activation of polo kinase on the spindle pole in the MPF-activating feedback loop.
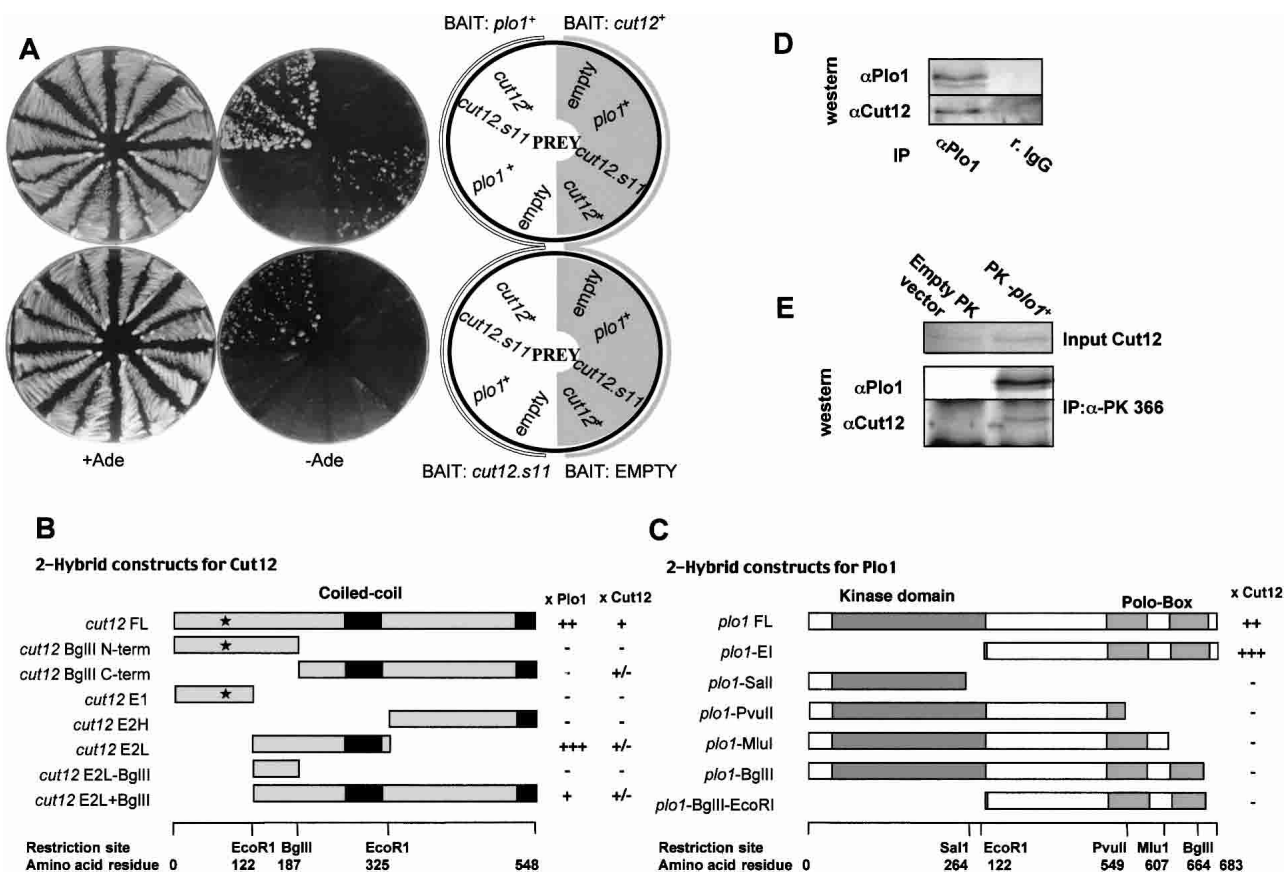

Figure 1. Cut12 and Plo1 associated in two-hybrid and immunoprecipitation assays. (*A*) Yeast strains (PJ69-4A) containing the indicated bait and prey plasmids were streaked on plates lacking or including adenine as indicated. (*B*) The truncations of the *cut12* gene represented in the cartoons were cloned into the prey vector and screened against the full-length *plo1+* bait vector. The site of the *cut12.s11* mutation is indicated by *. (*C*) *plo1* truncations cloned into the bait vector and screened against the full-length *cut12+* prey vector. (*B,C*) The strength of interaction with the molecule above the column is indicated by + symbols. The – symbol denotes no detectable interaction. The scale below indicates the restriction sites in the context of the amino acid sequence. (*D*) Immunoprecipitates were prepared from exponentially growing cells using either polyclonal antibodies to Plo1 or a nonspecific, preimmune serum (r.IgG), and the resulting blots were probed with Cut12 antibodies. (*E*) The monoclonal antibody MAb336, which recognizes the Pk epitope, was used to generate immunoprecipitates from extracts of a wild-type strain containing p41plo1.NPk (Tanaka et al. 2001) and/or an empty Pk vector cultured in the absence of thiamine to induce expression. Blots were probed with the antibodies indicated.

Results

Cut12 associates with Plo1

Yeast two-hybrid and immunoprecipitation assays were used to determine whether Plo1 bound directly to Cut12. *cut12⁺* and *plo1⁺* were each inserted into yeast two-hybrid bait and prey vectors (James et al. 1996). A positive reaction was seen when *plo1⁺* was cloned into the bait and *cut12⁺* into the prey plasmid, but not when *cut12⁺* was put in the bait and *plo1⁺* in the prey (Fig. 1A). A reduced interaction in one direction of the assay between partners is an established feature of this assay (Knop and Schiebel 1998). We also detected a weak interaction between Cut12 and itself (Fig. 1A). The generation of subclones established that the central region of Cut12 between amino acids 122 and 325 and the C-terminal 361 amino acids of Plo1, which contain the "polo boxes," were important for the interaction (Fig. 1B,C). We then asked whether Plo1 immunoprecipitates contained Cut12 protein. Cut12 coprecipitated with Plo1 when Plo1 was precipitated with either polyclonal anti-Plo1 antibodies (Fig. 1D), or when an epitope-tagged Plo1 protein was precipitated with antibodies to the tag (Fig. 1E). Thus, Cut12 and Plo1 interact in the two-hybrid assay and are present in common immunoisolated complexes, suggesting that they physically associate with one another.

The association of Cut12 with Plo1 prompted us to ask whether Cut12 function influenced Plo1 activity. We monitored the kinetics of Plo1-associated kinase activity in vitro and in vivo. We used established in vitro Plo1 kinase assays to measure Plo1-specific activity in immunoprecipitates (Tanaka et al. 2001), whereas the in vivo analyses relied on in situ recognition of phospho-epitopes by the monoclonal antibody MPM-2.

Cut12 is required for full activation of Plo1

We monitored Plo1 in vitro kinase activity during mitotic commitment in a *cut12.1* mutant strain. *cut12.1* cells divide normally at 25°C, but the loss of Cut12 function at 36°C blocks mitosis as cells form monopolar rather than bipolar spindles (Bridge et al. 1998). We used *cut7.24* to control for the possibility that any changes we observed were specific consequences of the loss of Cut12 function rather than general features of an early mitotic arrest. *cut7⁺* encodes a BimC-related mitotic motor protein that is required for spindle formation. Like *cut12.1*, *cut7.24* cells are unable to form a mitotic spindle and accumulate early in mitosis with condensed chromosomes (Hagan and Yanagida 1990).

Plo1-specific activity was monitored as the small G2 cells progressed through the cell cycle at either 25°C or
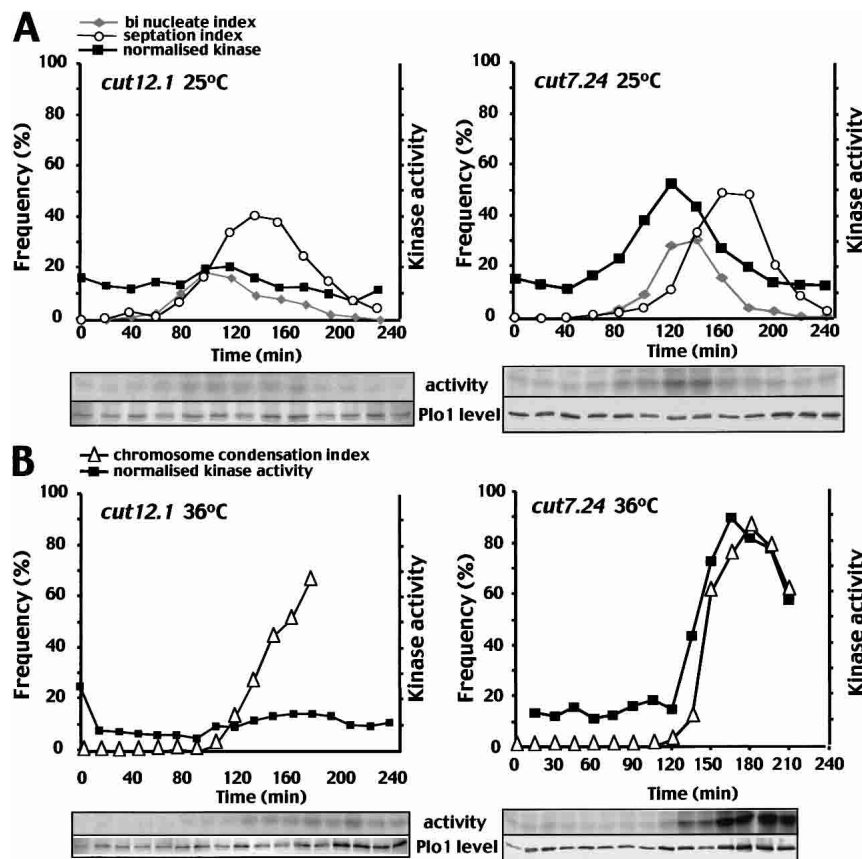

Figure 2. Plo1 kinase activity did not rise upon entry into mitosis in *cut12.1* mutants. Small G2 cells from mid-log phase cultures of *cut12.1* or *cut7.24* that had been grown in minimal medium at 25°C were incubated at either 25°C (*A*) or 36°C (*B*). Samples were processed for either Plo1 kinase assays or DAPI (chromatin) and calcofluor (cell wall) staining at the times indicated. The *top* panels show kinase assays, and the *bottom* panels show the amount of Plo1 in each kinase assay reaction. The septation and binucleate (25°C) or chromosome condensation (36°C) indices (*left*-hand *Y*-axis) were plotted alongside the kinase activity per unit Plo1 protein (i.e., specific activity, *right*-hand *Y*-axis). Assays were processed in an identical manner with identical cell numbers so that the arbitrary units used to plot the data on the graphs are comparable between the different panels.

36°C. *cut7.24* and *cut12.1* both resembled wild-type cells as Plo1-associated kinase activity rose and fell with cell division at 25°C (Fig. 2A; Tanaka et al. 2001). Plo1-associated activity did not rise to the same extent in *cut12.1* cells as it did in *cut7.24* or wild-type cells (Tanaka et al. 2001). At 36°C, the sharp rise in chromosome condensation indicated that *cut12.1* cells entered mitosis synchronously (Fig. 2B). However, this mitotic commitment was not accompanied by the surge of Plo1-associated kinase activity that is normally seen in the wild-type cells (Tanaka et al. 2001), or in the mitotically arrested cells in the control *cut7.24* culture. In *cut7.24* cells, Plo1-associated activity rose 10 min before the chromosomes condensed and continued to rise as cells accumulated in mitosis (Fig. 2B). No such mitotic stimulation of Plo1 activity occurred in *cut12.1* cells (Fig. 2B). Histone H1 kinase assays established that histone H1 kinase activity rose in a similar manner in the *cut12.1* and *cut7.24* synchronous cultures at both the permissive and restrictive temperatures (Fig. 3A,B). Thus, despite the lack of any increase in Plo1-specific activity at this time, histone H1 kinase activity rose normally as the chromosome condensation indices of the same samples indicated that these *cut12.1* cells were accumulating in mitosis (Fig. 3B). This suggested that the inability to promote mitotic Plo1-associated kinase activity in *cut12.1* cells was not a simple consequence of an inability to assemble a bipolar spindle, or an inability to commit to mitosis. Rather, the data indicate that Cut12 function was required for full activation of Plo1-associated kinase activity during mitotic commitment.

Activation of interphase Plo1 in vitro kinase activity by the cut12.s11 *mutation*

Having established the dependency of Plo1-associated kinase activity on Cut12 function, we next asked whether the dominant *cut12.s11* mutant led to any changes in the biochemical characteristics of Plo1. Plo1 levels remain constant throughout the cell cycle and were unaffected by the *cut12.s11* mutation (Fig. 4A; Mulvihill et al. 1999). Blotting extracts from an asynchronous *cut12.s11* culture revealed a single band at the same position and of the same intensity as wild-type Cut12, indicating that the *cut12.s11* mutation did not alter Cut12 levels (Fig. 4B). Nor did *cut12.s11* alter the ability of Cut12 to interact with Plo1 in two-hybrid or coimmunoprecipitation assays (Figs. 1A, 4C). We next assayed Plo1-associated kinase activity in Plo1 immunoprecipitates from *cut12.s11* cells in which cell cycle progression had been arrested in interphase by incubation of the conditional *cdc2* mutation *cdc2.33* at 35°C for 2 h. Plo1-associated kinase activity of extracts from arrested *cdc2.33 cut12.s11* cells was 2.4-fold (±0.35; $n = 6$) higher than that of the control *cdc2.33 cut12*+ cells (Fig. 4D). This established that *cut12.s11* increased Plo1 activity in interphase. Moreover the use of the *cdc2.33* mutant background established that MPF activity was not required to induce Plo1 activation in *cut12.s11* mutant cells. Returning the temperature of the same two populations to 25°C for 30 min promoted mitosis. Kinase assays of these mitotic samples indicated that the *cut12.s11* mutation promoted a 1.6 (±0.18; $n = 5$) in-
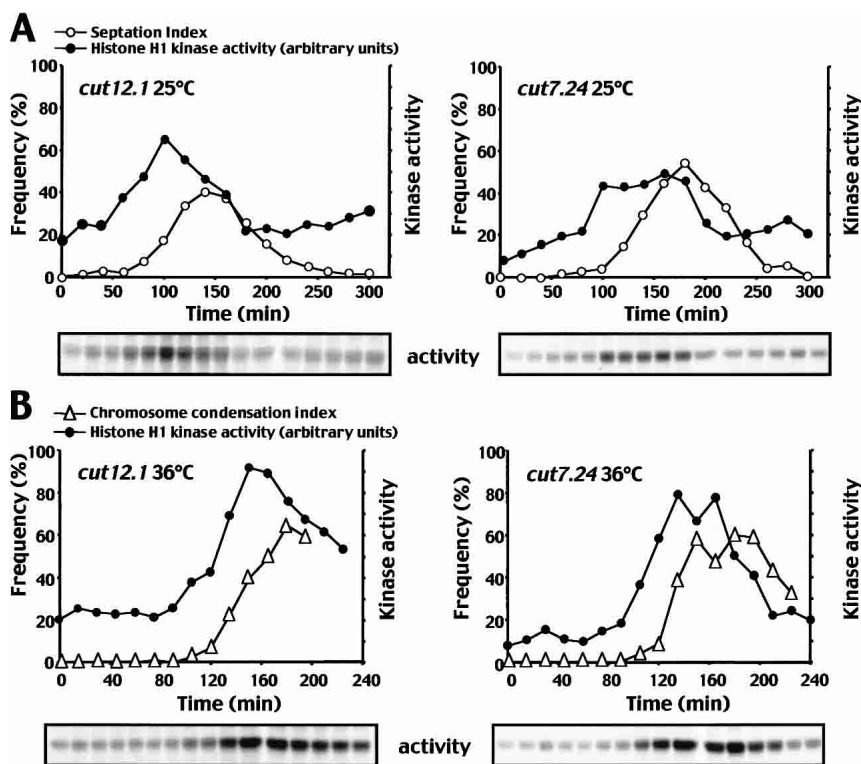
Figure 3. Histone H1 kinase activity rose as *cut12.1* cells entered a defective mitosis. Small G2 cells from mid-log phase cultures of *cut12.1* or *cut7.24* that had been grown in minimal medium at 25°C were incubated at either 25°C (*A*) or 36°C (*B*). Samples were processed for either histone H1 kinase assays or DAPI (chromatin) and calcofluor (cell wall) staining at the indicated times. The kinase assays are shown at the *bottom* of each panel. The septation (25°C) or chromosome condensation (36°C) indices (*left*-hand *Y*-axis) were plotted alongside the kinase activity (*right*-hand *Y*-axis). The kinase assays were processed in an identical manner with identical cell numbers.
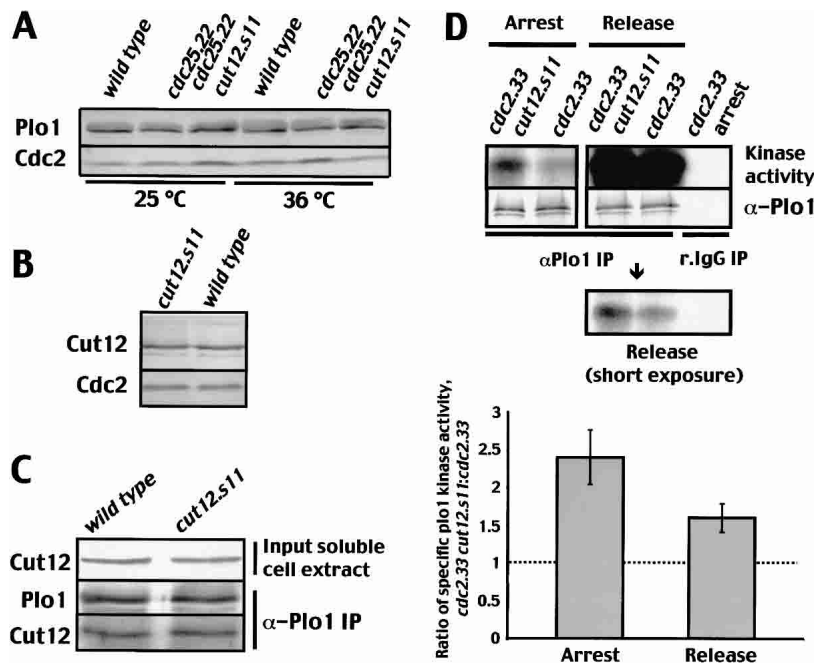
Figure 4. The *cut12.s11* mutation enhanced Plo1 kinase activity. (*A*) Cells were grown to early log phase at 25°C before the culture was split in two. One-half was incubated at 25°C and the other at 36°C for 4 h before protein extracts were processed to blot for Plo1. (*B*) Western blot showing Cut12 levels in *cut12⁺* and *cut12.s11* cells. (*C*) Western blot of *cut12⁺* and *cut12.s11* cultures showing Cut12 levels in the cell extracts (*top*) that were used to immunoprecipitate Plo1 (*middle*) and probe for coprecipitated Cut12 (*bottom*); for controls, see Figure 1D. (*D*) Plo1 kinase assays of *cdc2.33 cut12⁺* and *cdc2.33 cut12.s11* cells that had been grown in minimal medium at 25°C before shifting to 36°C for 2 h (*left* panel). Cells were then returned to 25°C, and a sample was taken from the mitotic fraction 30 min after the release (*right* panel). The *top* panel shows Plo1-associated kinase activity, and the panel directly *below* it shows the quantity of Plo1 in the immunoprecipitates. Immunoprecipitation was either with antibodies to Plo1 (αPlo1 IP) or nonspecific preimmune antiserum (r.IgG). All lanes in the kinase assays in the *top* panel were exposed to the same extent, but the *middle* panel in *D* shows a lower exposure of the mitotic samples following release from G2 arrest. The *bottom* panel shows a graph of the ratio of specific Plo1 kinase activity of the double *cdc2.33 cut12.s11* mutant to that of the single *cdc2.33* mutant.

crease in Plo1-specific activity during mitosis (Fig. 4D). Because Plo1 activation normally requires the prior activation of p34[cdc2] (Tanaka et al. 2001), these data support the hypothesis that *cut12.s11* suppresses *cdc25.22* because it promotes the activity of the MPF amplification loop kinase Plo1 in interphase cells and thereby promotes the autoamplification loop that drives mitotic commitment.

Recognition of the mitotic SPB by the phospho-specific antibody MPM-2 is dependent on Plo1 function

Having established that loss or gain of Cut12 function led to a corresponding loss or gain of Plo1 function in in vitro kinase assays, we asked whether this was due to particularly atypical behavior in in vitro assays, or whether it also held true in vivo. We used the monoclonal antibody MPM-2 to monitor in vivo Plo1-dependent events on the SPB. MPM-2 recognizes a mitosis-specific phospho-epitope that is greatly enriched at spindle poles (Davies et al. 1983; Vandre et al. 1984; Lane and Nigg 1996). MPM-2 reactivity of the spindle pole of HeLa cells is abolished by microinjection of antibodies to human polo kinase, Plk1, and phosphorylation by Polo kinase confers MPM-2 reactivity on Cdc25 (Kumagai and Dunphy 1996; Lane and Nigg 1996).

MPM-2 antibodies gave clear cell cycle stage-dependent staining patterns in *Schizosaccharomyces pombe* (Fig. 5). Signals from the spindle and cytokinetic ring could be seen over a faint nuclear background stain and small dots that could represent kinetochores (Fig. 5, row 4). The optimal conditions for staining particular struc-

tures varied. For example, cytokinetic ring staining (Fig. 5, row 7, arrow) was optimal after 8% formaldehyde for 30 min, whereas SPB staining was optimal after 3.7% formaldehyde for 2 min (data not shown). We frequently observed a line of MPM-2 staining between the two SPBs just before anaphase (Fig. 5, row 5, arrow). This may represent a chromosome topology protein such as the cohesin subunit and polo kinase target Rad21 (Weitzer and Uhlmann 2002). The strongest staining however, was of mitotic SPBs up to late anaphase. The SPB was identified by costaining with antibodies to the SPB component Sad1. The SPB stained strongly from commitment to mitosis when a single spot of SPB staining was visible (Fig. 5, row 3) until the middle of anaphase B, whereupon it became much fainter. This staining pattern is highly reminiscent of the patterns seen with anti-Plo1 antibodies (Mulvihill et al. 1999).

We asked whether MPM-2 reactivity was dependent on Plo1 activity by comparing MPM-2 staining in *plo1.ts2* (see below), wild-type, and *cut7.24* cultures after the cells had been incubated at 35°C for 4 h to inactivate Plo1 and Cut7, respectively. Both *plo1.ts2* and *cut7.24* mutants block spindle formation (see below). Because the short fixation times required to see MPM-2 epitopes did not preserve chromatin structure, parallel samples were processed for DAPI staining to determine the frequency of cells with either chromosome condensation or two nuclei in each sample. The chromosome condensation index of 49% indicated the accumulation of *cut7.24* cells in mitosis. This mitotic block resulted in a large increase in the proportion of cells in which the SPBs stained with MPM-2 from 11% to 71.5% (Fig. 6A,B). In
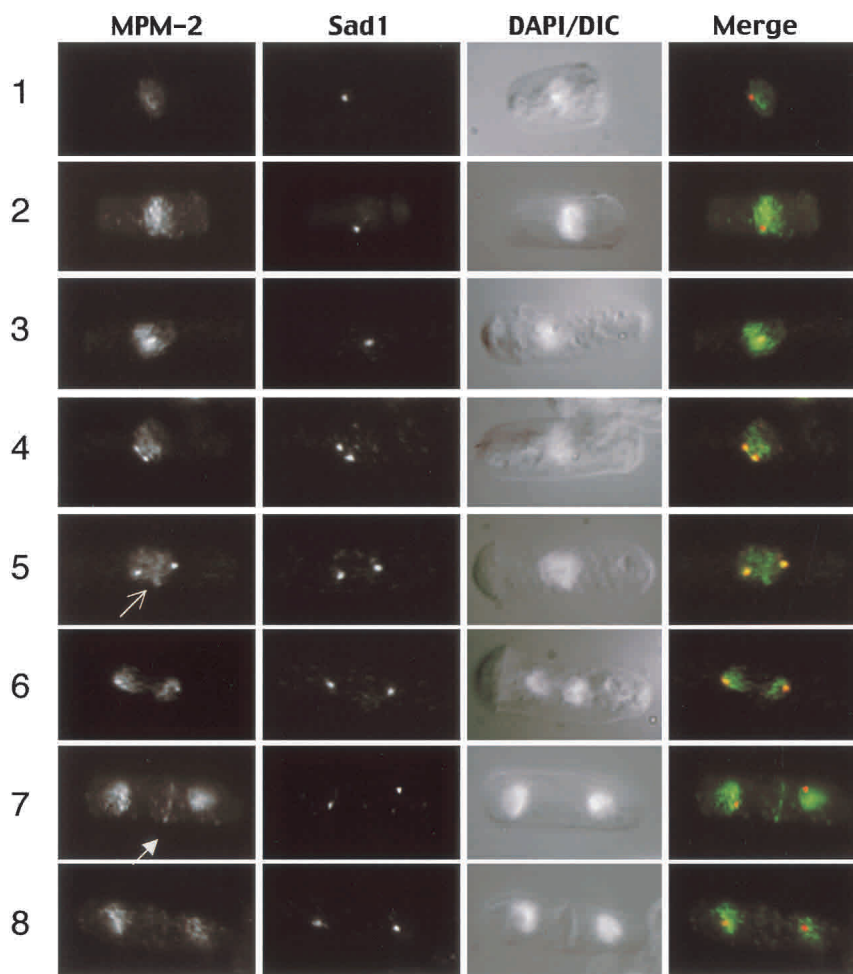
Figure 5. MPM2 recognizes the mitotic SPBs and a range of other structures during cell division. Wild-type cells were grown to mid-log phase at 25°C before processing for simultaneous visualization of Sad1, MPM-2, and DAPI staining. The signals for MPM2 (green) are merged with those for Sad1 (red) in the far *right* panel. The arrows in rows 5 and 7 indicate linear elements in the chromatin and the cytokinetic ring, respectively. All cell cycle stages are shown: interphase (rows 1,2), prophase (row 3), prometaphase (row 4), metaphase (row 5), anaphase B (row 6), telophase (row 7), and G1/cytokinesis (row 8).

contrast, even though the chromosome condensation index of 21.5% showed a strong accumulation of *plo1.ts2* cells in mitosis, there was no concomitant increase in MPM-2 reactivity of the SPBs (Fig. 6A,B). This established that the MPM-2 reactivity of mitotic SPBs was dependent on the activity of Plo1 kinase.

A direct correlation between MPM-2 recognition of the mitotic SPB and loss or gain of Cut12 function

Inactivation of Cut12 by shifting the temperature of a *cut12.1* culture from 25°C to 35°C led to an accumulation of cells blocked in mitosis with a chromosome condensation index of 24.5% (Fig. 6B). Despite this accumulation in a phase of the cell cycle during which SPBs become MPM-2 reactive, we detected no concomitant increase in the number of MPM-2 reactive SPBs in this *cut12.1* culture (Fig. 6B). This result mirrored the reduction in Plo1 in vitro kinase activity in *cut12.1* (Fig. 2B) as it showed that an in vivo readout of Plo1 activity was dependent on Cut12 function. We next asked whether the enhancement of in vitro Plo1-associated kinase activity in *cut12.s11* mutants (Fig. 4D) was similarly mirrored by an increase in Plo1-dependent MPM-2 phos-

phorylation in vivo. Excitingly, it was (Fig. 6B). *cut12.s11* led to a 1.8 ± 0.05-fold increase in the number of MPM-2 reactive SPBs at 25°C and 2.0 ± 0.24-fold at 35°C (Fig. 6C). This increase was Plo1-dependent because no MPM-2 staining was seen when Plo1 was inactivated at 35°C in *cut12.s11 plo1.ts2* double-mutant cells (Fig. 6B). Most importantly, there was no difference between the number of MPM-2 reactive SPBs of wild-type and *cut12.s11* cells at 37°C (Fig. 6C). This is highly significant because it is well established that *cut12.s11* is unable to suppress *cdc25.22* at this temperature (Hudson et al. 1990; Bridge et al. 1998).

In summary, when *cut12.s11* was able to suppress *cdc25.22*, it also enhanced a Plo1-dependent phosphorylation event on the SPB. When it was no longer able to suppress *cdc25.22*, it no longer enhanced this phosphorylation event. Furthermore, the phosphorylation event did not happen in the loss-of-function mutant *cut12.1*. This mutation both blocked the mitotic stimulation of Plo1 in vitro kinase activity and has shown a very strong synthetic enhancement of the *cdc25.22* phenotype (Bridge et al. 1998). We can make two key observations from these data. First, there is a striking correlation between Cut12 function and Plo1-dependent phosphoryla-
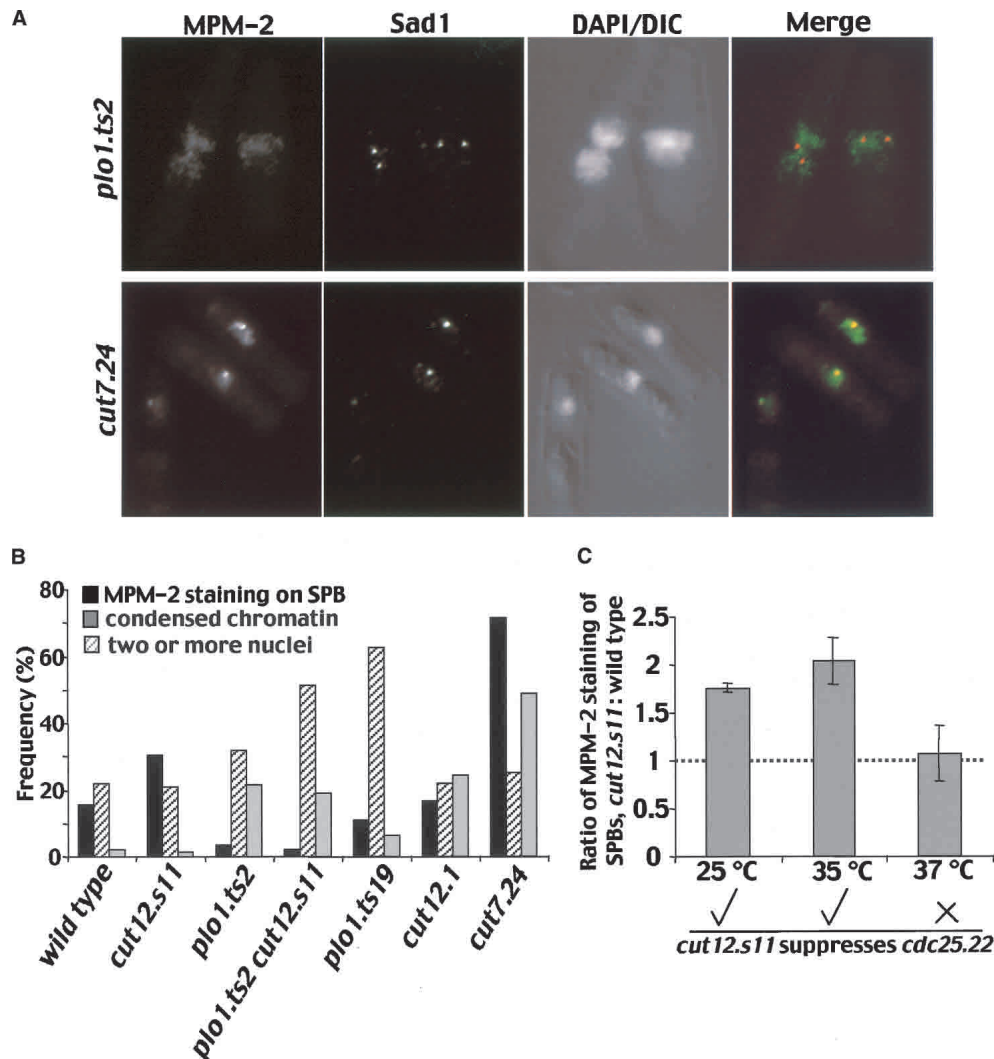
Figure 6. MPM2 recognition of mitotic SPBs depended on Plo1 and Cut12 function and was enhanced in *cut12.s11* mutants when it could suppress *cdc25.22*. (*A,B*) MPM-2/Sad1 staining of *plo1.ts2* and *cut7.24* cells that had been incubated at 35°C for 4 h as described for Figure 4. (*B*) A bar chart of the frequency of cells with the features shown in the legend. (*C*) The ratio of the frequency of MPM-2 recognition of SPBs in *cut12.s11* over wild type at the temperatures shown. The ticks and cross underneath the graph indicate whether *cut12.s11* can (√) or cannot (✗) suppress *cdc25.22* at this temperature.

tion events at the SPB. Second, *cut12.s11* enhances Plo1 activity under conditions in which it also suppresses *cdc25.22*.

Does the increase in Plo1 activity in cut12.s11 *account for its ability to suppress* cdc25.22*?*

We next wanted to ask whether the correlation between the ability of *cut12.s11* to both induce increased Plo1 activity and suppress *cdc25.22* reflected a mechanistic link between enhancement of Plo1 activity and the suppression of *cdc25.22*. In other words, did *cut12.s11* suppress *cdc25.22* because it increased Plo1 activity in interphase? To address this question, it was necessary to generate conditional *plo1* mutants that could be introduced into *cut12.s11 cdc25.22* mutants to ask whether

compromising Plo1 activity would stop *cut12.s11* from promoting mitosis of *cdc25.22*.

plo1.ts2 *and* plo1.ts19 *have cell division defects*

Two temperature-sensitive *plo1*mutants were generated via a marker switch approach (MacIver et al. 2003). *plo1.ts2* has the change E139K in the kinase domain, whereas *plo1.ts19* introduced a premature stop codon after the kinase domain, but before the polo boxes, at position 316 (Fig. 7A). The temperature sensitivity of *plo1.ts19* was suppressed by expression of the opal nonsense suppressor tRNA *sup3.5* (data not shown; Hawthorne and Leupold 1974). Both *plo1.ts2* and *plo1.ts19* conferred temperature sensitivity for growth on minimal medium. In contrast, the ability of *plo1.ts19* cells to
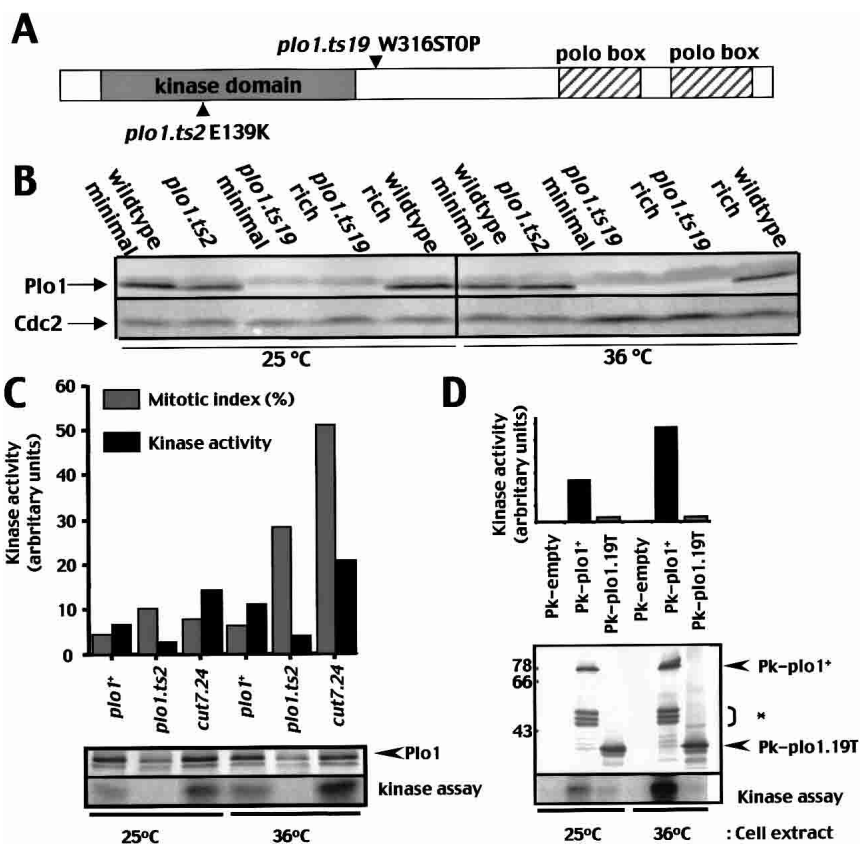
Figure 7. Plo1 kinase activity was severely reduced in *plo1.ts2* and *plo1.ts19*. (*A*) A cartoon indicating the position of the mutations in the *plo1* gene. (*B*) Mid-log-phase cultures of cells grown in either rich or minimal medium were prepared for Western blot analysis to detect Plo1 or p34[cdc2] at the temperatures indicated. (*C,D*) Plo1 kinase assays of wild-type cells containing the plasmids indicated on the graphs after growth to mid-log phase in minimal medium at the indicated temperatures. The level of Plo1 kinase activity per unit of kinase in the precipitate (i.e., specific activity) was plotted to generate the graphs.

form colonies on rich medium at 36°C was indistinguishable from that of wild-type cells. The introduction of a single *plo1+* gene at the *leu1* locus restored wild-type characteristics to either mutant, indicating that they were recessive, loss-of-function mutations (data not shown). Western blot analysis showed that Plo1 levels in *plo1.ts2* cells were not radically different from wild type, whereas the full-length protein appeared to be largely absent from *plo1.ts19* on either minimal or rich medium at either 25°C or 36°C (Fig. 7B). Plo1-associated kinase in vitro kinase activity was greatly reduced in extracts from *plo1.ts2* that were prepared at either 25°C or 36°C (Fig. 7C).

The premature stop codon in *plo1.ts19* removed the epitopes recognized by the polyclonal antibodies that were routinely used for the Plo1 kinase assays (Ohkura et al. 1995). We therefore generated a version of the *plo1* gene in which a stop at position 316 (equivalent to the *plo1.ts19* mutation site) was directly followed by the *nmt1+* termination sequences. The N terminus of this molecule was fused to the Pk tag, and it was expressed from the derepressed weakened *nmt1+* promoter of pREP81 (pREP81NPkplo1.19T). Induction of gene expression of the tagged plo1.19T or the wild-type control molecule for 30 h did not affect progression through mitosis (data not shown). Anti-Pk tag antibodies were used to generate immunoprecipitates from strains that had been expressing either tagged *plo1.19T* or tagged *plo1+*, alongside the product of the native *plo1+* gene for 30 h.

The kinase activity associated with the mutant precipitates was barely higher than the empty vector control and much lower than that seen in wild-type extracts. This was true whether the protein was isolated from cells grown at 25°C or 36°C or the assays were done at 30°C or 36°C (Fig. 7D; data not shown). We concluded that the Plo1-dependent kinase activity of both *plo1.ts2* and *plo1.ts19* was greatly reduced.

plo1.ts2 strains entered mitosis but did not form spindles as multiple microtubule bundles extended from a single focus of staining for the spindle pole marker Sad1 (Hagan and Yanagida 1995; upper two panels in Fig. 8A; Table 1). Unlike classic "cut" mutants (Hirano et al. 1986), septation did not always follow on from the mitotic arrest. In the cells that did septate, 95.5% of the septa were misshapen (Fig. 8A, lower right panel). *plo1.ts19* cells had a range of defects, including monopolar spindles (Fig. 8B, left-hand cell in the series of images; Table 1). Some *plo1.ts19* cells did not form a septum and became bi- and multinucleated (Fig. 8B, lower left panel). These deficiencies in septation presumably arose from an inability to activate the septum initiation network (Tanaka et al. 2001).

plo1.ts2 *and* plo1.ts19 *show full activation of histone H1 kinase*

Histone H1 kinase activity gives a good assay of MPF activity and the timing of mitotic commitment in *S.*
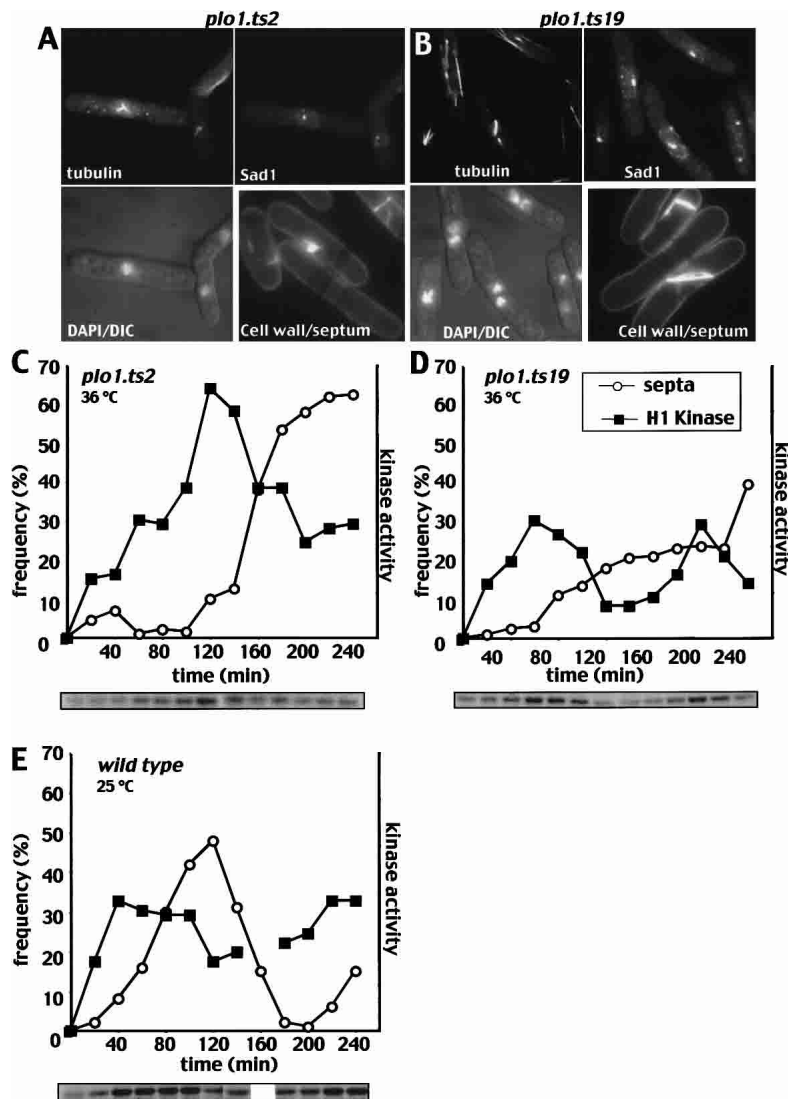
Figure 8. Histone H1 kinase activity increased as *plo1.ts2* and *plo1.ts19* cells underwent defective cell divisions. (*A,B*) *plo1.ts2* and *plo1.ts19* cultures were grown to early log phase in minimal medium at 25°C before the temperature of the culture was shifted to 36°C for 3 h. One portion was then stained for tubulin, the spindle pole marker Sad1, and DAPI to give the *top* two and *bottom left*-hand panels. The cell wall and septum were stained with calcofluor in a second portion. (*C–E*) H1 kinase and septation profiles of cultures of the indicated strains. After isolation from a culture that was grown in minimal medium at 25°C, small G2 cells were incubated at the temperatures indicated.

pombe (Moreno et al. 1989; Creanor and Mitchison 1994). We therefore used H1 kinase assays of extracts from *plo1* mutant cells, synchronized with respect to

cell cycle progression, to ask whether the kinetics of mitotic commitment were affected by mutation of *plo1⁺*. Both *plo1.ts2* and *plo1.ts19* cells entered mitosis and activated histone H1 kinase. H1 kinase accumulated at a similar rate over a longer period in *plo1.ts2* than in either *plo1.ts19* or wild type. The delay in the appearance of septa and the accumulation of cells in mitosis indicated that the defect in spindle formation in *plo1.ts2* activated the spindle checkpoint (Fig. 8C–E; Table 1). In contrast, the septation defect of *plo1.ts19* did not activate any mitotic checkpoints, so that H1 kinase activity in this strain was periodic and a second wave of activity accompanied a second attempt at division at 36°C (Fig. 8D, 220 min).

Plo1 is required for the suppression of cdc25.22 *by* cut12.s11

We were now in a position to use *plo1.ts2* and *plo1.ts19* to ask whether full Plo1 function was required for the dominant *cut12.s11* mutation to promote transition of

Table 1. *Frequencies of mitotic phenotypes (%) in* plo1.ts2 *and* plo1.ts19 *after 3 h at 36°C*

	plo1⁺	*plo1.ts2*	*plo1.ts19*
Mitotic index	8.5	27.0	19.5
Proportion of mitotic cells with monopolar spindles	0	94.5	50.0
Proportion of binucleate cells in mitosis that have monopolar rather than bipolar spindles	0	5	59
Metaphase spindles	27.0	0	50.0
Septation index	12.0	34	24
Proportion of septa that are disorganized or misplaced	0	95.5	86.5
Cells with two or more nuclei	22	10.5	70.5

the G2/M boundary when Cdc25 function was compromised. If the suppression of the cell cycle regulator by mutation of the spindle pole component was due to an enhancement of Plo1 function, then compromising Plo1 should compromise the ability of *cut12.s11* to suppress *cdc25.22*. In other words, triple *cut12.s11 cdc25.22 plo1.ts* mutant cells should not enter mitosis because they arrest cell cycle progression at the G2/M boundary.

In a first assay, we took advantage of the media dependence of the *plo1.ts19* phenotype. On rich medium at 36°C, *plo1.ts19* cells formed colonies with a proficiency that was indistinguishable from either wild type or *cut12.s11* controls (Fig. 9A). *cdc25.22* and *cdc25.22 plo1.ts19* cells, on the other hand, could not form colonies on this medium at this temperature, but *cdc25.22 cut12.s11* cells could (Hudson et al. 1990; Bridge et al. 1998). Spot test analysis showed that although the *plo1.ts19* mutation did not affect colony formation of *cut12+*, triple *cut12.s11 cdc25.22 plo1.ts19* cells were unable to grow (Fig. 9A). This established that mutating

plo1 in a way that did not affect cell viability compromised the ability of *cut12.s11* to suppress *cdc25.22*.

In a second assay, we asked whether the introduction of the *plo1.ts19* or *plo1.ts2* mutations would affect the ability of *cut12.s11 cdc25.22* to enter mitosis at 36°C. Because neither *plo1.ts2* nor *plo1.ts19* by itself affected the full-scale activation of MPF (Fig. 8), an effect of these mutations on the suppression of *cdc25.22* by *cut12.s11* would indicate that *cut12.s11* was reliant on Plo1 function to suppress *cdc25.22*. Cells were grown in minimal medium and shifted to 36°C for 3 h. Whereas single *plo1.ts2* and *plo1.ts19* mutant and double *cut12.s11 cdc25.22* mutant cells all entered mitosis (Fig. 9B,C), the single *cdc25.22* mutant and both double *cdc25.22 plo1.ts* and triple *cut12.s11 cdc25.22 plo1.ts* mutants did not. These latter strains all arrested cell cycle progression with an interphase array of microtubules and no mitotic spindles (Fig. 9B,C). These data established that *cut12.s11* required full Plo1 function to transit the G2/M transition when Cdc25 function was compromised.
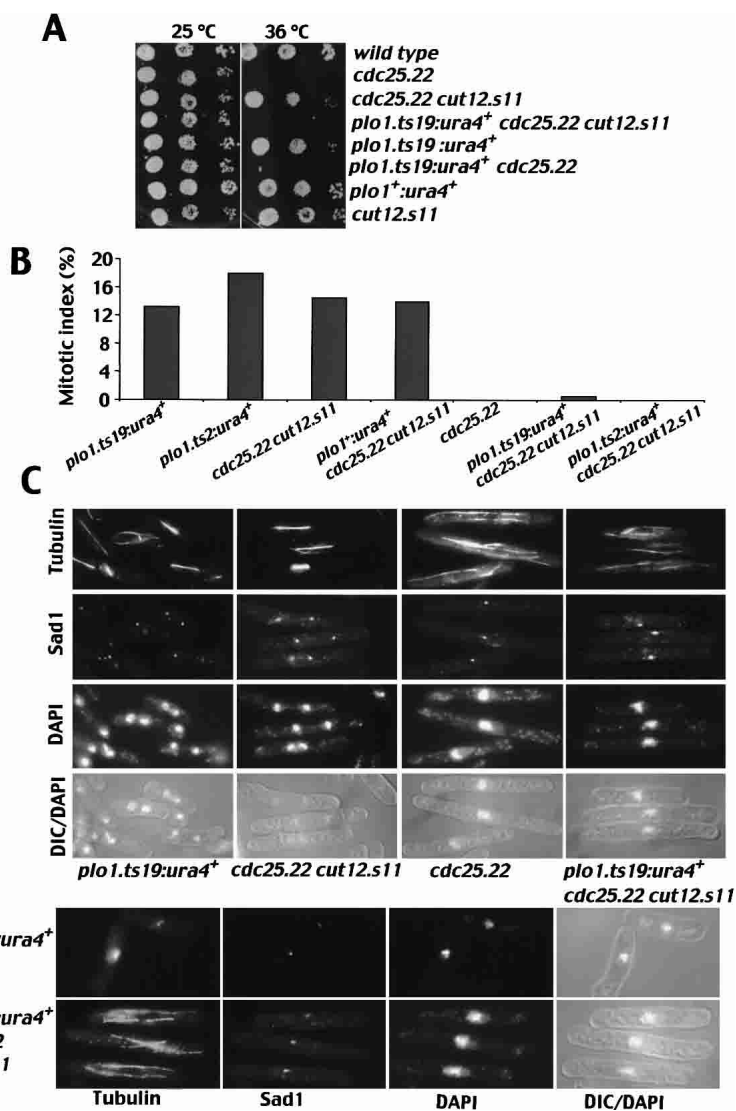

Figure 9. Plo1 function was required for *cut12.s11* suppression of *cdc25.22*. (*A*) A drop test analysis of the colony-forming potential of the indicated strains at 36°C on YES rich medium. Note that the *plo1.ts* mutants were generated by a marker switch approach that leaves a *ura4+* gene 235 bp upstream in the promoter. This is indicated by *plo1.x:ura4+*, and the wild-type equivalent allele is *plo1+:ura4+*. (*B,C*) Asynchronous early log-phase cultures of the seven strains shown in the figure were shifted from 25°C to 36°C and maintained at 36°C for 3 h before cells were processed for microscopy to simultaneously stain for tubulin, the spindle pole marker Sad1, and DAPI. (*B*) The mitotic index. (*C*) Representative images of each strain.

*Activation of Plo1 suppresses the mitotic commitment
defect of* cdc25.22 *cells*

If *cut12.s11* does promote suppression of *cdc25.22* by
boosting Plo1 activity in interphase cells, then it should
be possible to suppress *cdc25.22* by boosting Plo1 activity independently of the status of Cut12. Gross overproduction of Plo1 protein in *cdc25.22* cells promotes cytokinesis events that randomly bisect the nucleus and so
presumably activate the DNA damage checkpoint to inhibit mitotic commitment (Ohkura et al. 1995). We
therefore sought a less drastic means of enhancing Plo1
activity to address this issue. Mutation of the conserved
residues S128 and T201 to aspartic acid in *Xenopus* Plx
results in constitutive activity of this polo kinase (Qian
et al. 1999). We therefore introduced the analogous mutations (S124D T197D) into a version of the *plo1* gene
that was integrated at the *leu1* locus under the control of
an inducible promoter. The first Plo1 protein to be seen
following induction of *plo1* alleles from this promoter at
the *leu1* locus appears at the 13-h time point and takes 7
h to accumulate to the same level as the native protein
(Tanaka et al. 2001).

We asked whether *plo1.S124DT197D* affected interphase Plo1 activity by measuring Plo1 activity in a
cdc2.33 mutant in which the *plo1.S124DT197D* allele
was either repressed or induced. Figure 10 shows Plo1 in
cdc2.33 cells in which *plo1.S124DT197D* had been induced at 25°C for 13 h before incubation at the restrictive temperature of 35°C for 2 h had specific kinase ac-

tivities that were 8.7-fold higher than in the repressed
control (Fig. 10A,B). We next asked whether induction of
this mutant allele would promote mitosis of *cdc25.22*
cells at the restrictive conditions. We split a 25°C
cdc25.22 plo1.S124DT197D double-mutant culture in
two, induced *plo1.S1224DT197D* expression in one half,
and 22.5 h later shifted both cultures to 34°C for 3 h
before processing them to stain for microtubules, spindle
poles, and chromatin. Whereas cells in which the expression of the constitutively active mutant remained repressed arrested cell cycle progression in interphase,
11% of those in which it had been expressed entered
mitosis. This degree of suppression is very similar to the
level of suppression of *cdc25.22* arising from the presence of the *cut12.s11* mutation (Fig. 9B) and established
that activation of Plo1 is sufficient to suppress the deficiency in Cdc25 function in *cdc25.22* cells.

*Mitotic commitment was delayed and less tightly
regulated in* plo1.ts2

Did the ability of constitutively active Plo1 kinase to
suppress *cdc25.22* reflect a genuine requirement for Plo1
during mitotic commitment in an otherwise unperturbed cell cycle? The extended and more random size of
plo1.ts2 cells at division suggested that this may be the
case. Despite the fact that these cells are able to enter
mitosis, they appear to be doing so in a less efficient, or
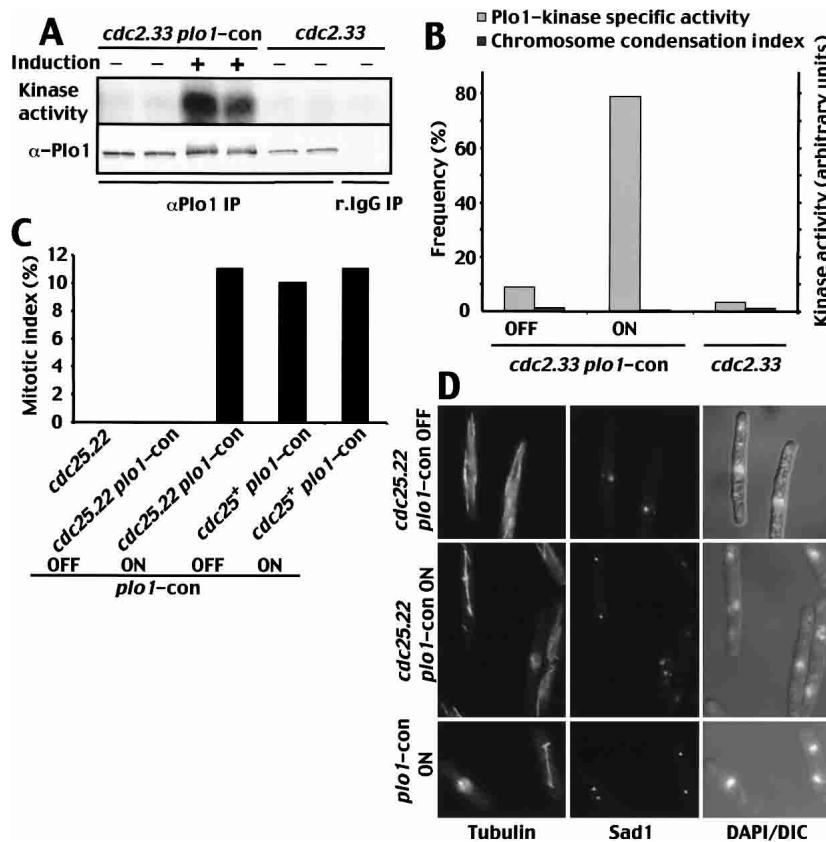more random manner (Fig. 8B). We therefore compared

Figure 10. Expression of a constitutively
active *plo1* mutant suppressed the mitotic
entry defect of *cdc25.22*. (*A,B*) A *cdc2.33
plo1.S124DT197D* (labeled *plo1.con* in the
figure) culture was grown in minimal medium containing thiamine at 25°C and split in
two, and one-half was washed and resuspended in thiamine-free medium to induce
plo1.S124DT197D transcription. Thirteen
hours later, both halves of the culture were
shifted to 35°C for 2 h before being processed
for in vitro Plo1 kinase assays (*A*) and scored
for chromosome condensation index by DAPI
staining (*B*). The specific activity per unit protein precipitated was calculated from the gels
and blot shown in *A* and plotted alongside the
chromosome condensation index in *B*. (*C–D*)
A *cdc25.22 plo1.S124DT197D* culture was
grown in minimal medium containing thiamine at 25°C and split in two, and one-half
was washed and resuspended in thiamine-free
medium to induce *plo1.S124DT197D* transcription. Then, 22.5 h later, both halves of
the culture were shifted to 34°C for 3 h before
being processed for immunofluorescence microscopy to visualize microtubules (*top left*
panel), Sad1 (*middle* panel), or DAPI/DIC
(*bottom right* panel).

Table 2. *Cell length at division*

Strain	25°C	36°C
Wild type	13.5 ± 1.1	13.8 ± 0.9
plo1.ts2	13.4 ± 1.3	17.6 ± 3.22
cut7.24	13.2 ± 0.9	15.4 ± 1.11
cut12.1	14.6 ± 1.3	15.0 ± 0.8

Cell length measurements (µm) of cells with condensed chromatin or anaphase nuclei were taken after staining with DAPI.

the length of mitotic *plo1.ts2* cells with wild type and *cut7.24* controls. Cells were stained with DAPI, and the length of all cells that had condensed chromatin or anaphase nuclei was recorded (Table 2). Wild-type cells entered the mitotic, constant-length phase (Mitchison and Nurse 1985) at 13.8 ± 0.9 µm, *cut7.24* at 15.4 ± 1.11 µm, and *plo1.ts2* at 17.6 ± 3.22 µm. Thus, mitotic commitment was later and not as stringently controlled in *plo1.ts2* than in either of the control strains. In other words, although mutation of *plo1* did not affect the ability to enter mitosis per se, the timing of mitotic commitment was heavily influenced by the status of Plo1.

Mutant Plo1 does not associate with the interphase SPB of cut12.s11

We next asked whether *plo1.ts2* or *plo1.ts19* affected the affinity of Plo1 for the interphase SPB of *cut12.s11* cells.

For this assay, cells were first incubated in hydroxyurea (HU) for 5 h to generate an interphase population in which cell cycle progression was arrested in early S phase. As described previously, Plo1 did not show strong affinity for the SPB of wild-type cells under these conditions, but did associate with virtually all of the SPBs of *cut12.s11* cells at all temperatures (Fig. 11A,B,E; Mulvihill et al. 1999; Grallert and Hagan 2002). At 25°C, *plo1.ts2 cut12.s11* resembled *cut12.s11* as Plo1 associated with both interphase and mitotic SPBs (Fig. 11A,C). In contrast at 36°C, Plo1 did not associate with interphase SPBs of this double mutant (Fig. 11E). Mixing a *cut12.s11* culture with the double mutant so that 25% of the cells in the mixture were *cut12.s11* and 75% were *cut12.s11 plo1.ts2* established that this lack of staining was not an artifact arising from a processing error as 20% of cells had Plo1 on their SPB (Fig. 11D,E).

The inability of a Plo1 mutant protein that has compromised Plo1-associated kinase activity to associate with the interphase SPB of *cut12.s11* raised the possibility that Plo1 activity may be critical for the recruitment of this kinase to interphase SPBs of *cut12.s11* cells. We therefore used anti-tag antibodies to compare the distribution of wild-type and "kinase dead" (*plo1.K65R*) molecules that were tagged at their N termini and expressed from the derepressed weakened *nmt1⁺* promoter of pREP81 (Tanaka et al. 2001). Whereas a similarly expressed tagged wild-type control molecule associated with both interphase and mitotic SPBs of *cut12.s11*,

Figure 11. *plo1.ts2* conferred temperature sensitivity on the ability of Plo1 to be prematurely recruited to the interphase SPB of *cut12.s11* cells. Ten millimolar HU was added to early-log-phase cultures of *plo1.ts2*, wild-type, *cut12.s11*, or *cut12.s11 plo1.ts2* cells that were growing in minimal medium at 25°C. Three hours later, each culture was split in two and one-half was incubated at 36°C and the other at 25°C for a further 2 h. After this, an extra culture was generated at 36°C by mixing the 36°C *cut12.s11* culture with the 36°C *plo1.ts2 cut12.s11* culture at a ratio of 1:3. All cultures were then processed for anti-Plo1 immunofluorescence microscopy. (*A,B*) Representative images from the *cut12.s11* culture at 25°C (*A*) and 36°C (*B*). (*C,D*) The mixed culture at 25°C (*C*) and 36°C (*D*). (*E*) A bar chart of the frequency of cells with Plo1 staining of the SPB. Plo1 associated with the SPB of *plo1.ts2 cut12.s11* cells at 25°C but not 36°C. The mixed culture (*plo1.ts2 cut12.s11* mixed with *plo1⁺ cut12.s11* cells) showed that the lack of staining in the *cut12.s11* strain was not due to processing artifacts. The 1:3 ratio of single to double mutants was reflected in the 20% of cells with Plo1 on the SPB. An example of Plo1 staining in the *cut12.s11* control cell in this mix is marked by * in *D*.
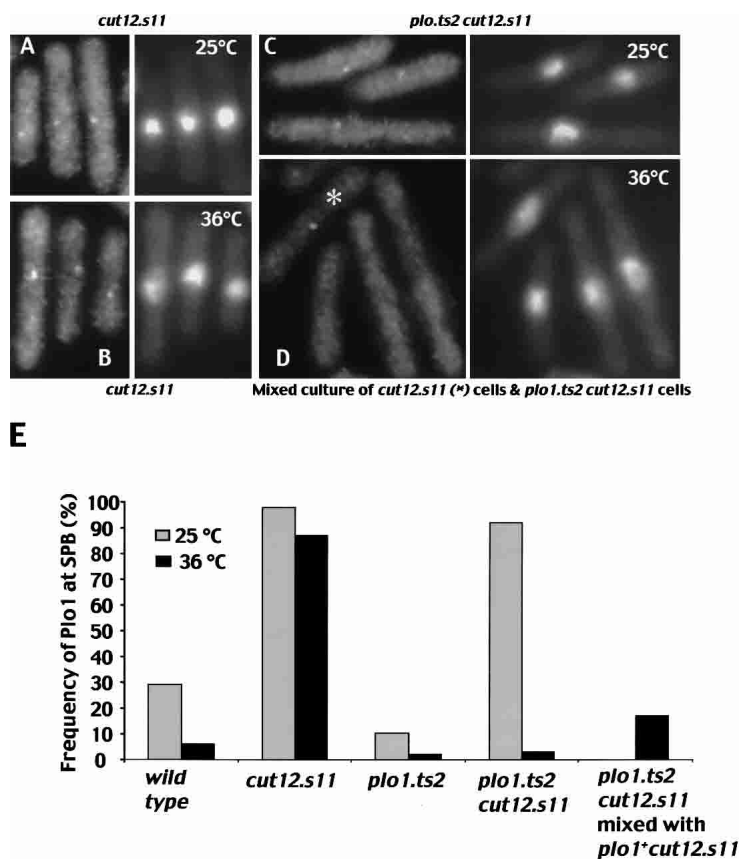
Plo1.K65R, the "kinase dead" mutant protein, only associated with mitotic but not with interphase SPBs (data not shown).

Anti-Pk antibodies were used to localize the truncated Plo1.19T protein that was expressed from pREP81NPkplo1.19T. Although the protein was expressed to similar levels to a full-length control (Fig. 7D; data not shown), no localization was seen at any stage of the cell cycle. This was consistent with the two-hybrid data that indicated that Plo1 associated with Cut12 via the C-terminal 361 amino acids and the failure of other premature truncation proteins to associate with the SPB (Reynolds and Ohkura 2003). We concluded that the efficient association of Plo1 with the interphase SPB of *cut12.s11* cells required both Plo1 activity and the C-terminal 367-amino-acid, polo-box-containing, region.

Discussion

Mutations in the spindle pole component Cut12 compensate for compromised Cdc25 function

In fission yeast, Cdc25 is absolutely required for mitotic commitment unless one of the following is mutated: the target p34^{cdc2}, the antagonizing kinase Wee1, or the spindle pole protein Cut12. In the absence of inhibitory kinase activity, newly synthesized p34^{cdc2} will form an unrestrained complex with cyclin B. Thus, as soon as the Wee1 homolog Mik1 is degraded at the end of S phase (Baber-Furani et al. 2000; Christensen et al. 2000), active MPF will accumulate and drive *wee1* cells into mitosis. Cdc25 is not therefore required to dephosphorylate MPF in Wee1 mutants because the active complex forms de novo as fresh cyclin B becomes available to partner p34^{cdc2}. Thus, it is fairly apparent why mutating *wee1* or the target *cdc2* can alleviate the requirement for Cdc25. It has not been so clear why the mutation of an SPB component should suppress *cdc25.22* and support colony formation of *cdc25Δ* (Hudson et al. 1990).

How could an altered spindle pole suppress cdc25.22?

Several models could account for *cut12.s11* suppression of *cdc25.22*. The site of the activating mutation in *cut12.s11* (G71V) lies 11 residues from an MPF consensus phosphorylation site and 4 residues from a MAP kinase consensus site, raising the possibility that the *cut12.s11* mutation may mimic the change induced by phosphorylation at one or both of these sites. Assuming that Cut12 was the major rate-limiting MPF substrate for mitotic entry, then *cut12.s11* may behave as an activated form of the protein and promote mitosis in the presence of reduced MPF activity. Introduction of plasmid-borne *cut12* with mutations in either, or both, the MAP kinase, or MPF consensus sites that may mimic the phosphorylated state failed to suppress *cdc25.22*. In contrast, plasmids with the *cut12.s11*, G71V, mutation, either alone or in combination with alanine or glutamic acid mutations at the MPF and MAP kinase consensus

sites, did suppress *cdc25.22* (K. Tanaka, A.J. Bridge, and I.M. Hagan, unpubl.). It therefore seems unlikely that *cut12.s11* is suppressing *cdc25.22* because it is mimicking activation of the sole essential MPF target.

MPF associates with the SPB (Alfa et al. 1990; Decottingnies et al. 2001). *cut12.s11* could therefore alter the recruitment of MPF to promote mitosis in *cdc25.22* cells; however, the lack of any effect of the *cut12.s11* or *cut12.1* mutations on the distribution of MPF supports the argument against this (Decottingnies et al. 2001; data not shown).

In a further model, Cut12 may influence mitotic commitment in *cdc25.22* through premature promotion of the feedback loop to counteract Wee1 function. Given that polo kinase is part of the feedback loop in *Xenopus* extracts, the simplest way by which Cut12 could influence this regulatory pathway would be by enhancing polo kinase activity.

Direct correlation between Cut12 function and Plo1 kinase activity and the suppression of cdc25.22

We have previously shown that *cut12.s11* influences the behavior of the fission yeast polo kinase, Plo1. Plo1 recruitment to the SPB is usually restricted to mitosis. In *cut12.s11* cells, however, it associates with the SPB throughout the cell cycle (Mulvihill et al. 1999; Grallert and Hagan 2002). We have now established that Cut12 associates with Plo1 in two-hybrid and immunoprecipitation assays. In vitro assays of Plo1 kinase activity showed that Plo1 activity depended on Cut12 function and that the gain-of-function mutation in *cut12.s11* enhanced Plo1 activity. Microscopy with MPM-2 antibodies that recognize Plo1-dependent phospho-epitopes suggested that the same held true in vivo. Loss-of-function mutants reduced MPM-2 staining, whereas gain-of-function mutants increased it. Two key observations make it unlikely that the lack of staining in *cut12.1* loss-of-function mutants arose from a general, nonspecific defect in SPB structure. First, a gain-of-function mutation in the same gene promotes a Plo1-dependent increase in the MPM-2 reactivity of SPBs. Therefore, the loss of recognition in a loss-of-function mutant (*cut12.1*) is directly mirrored by a gain of reactivity in a gain-of-function mutation in the same gene (*cut12.s11*). Second, there is an identical correlation between loss and gain of Cut12 function and the loss and gain of Plo1 activity in in vitro Plo1 kinase assays. Thus, we believe that there is a physical and functional link between Plo1 activity and the function of the spindle pole component Cut12. Crucially, the gain-of-function mutation *cut12.s11* was only able to promote Plo1-dependent phosphorylation at the SPB at temperatures at which it was also able to suppress *cdc25.22*. At 37°C, it was not able to either suppress *cdc25.22* or enhance Plo1-dependent phosphorylation of the SPB. This direct correlation between the promotion of Plo1 activity and suppression of *cdc25.22* suggested that the suppression of *cdc25.22* could be a direct consequence of the increase in Plo1 activity. The requirement for full Plo1 function for mitotic commitment in

cut12.s11 cdc25.22 cells suggests that this is, indeed, the case. However, although we cannot rule out the possibility that Plo1 may act in a parallel pathway, the body of data that links Cut12 and Plo1 suggests that a function in a parallel pathway is unlikely.

A critical prediction of a model whereby *cut12.s11* suppresses *cdc25.22* by promoting Plo1 activity is that promoting Plo1 activity independently of *cut12* mutations should also promote mitosis in *cdc25.22*. This was the case. We could induce the same degree of mitotic commitment in arrested *cdc25.22* cells by inducing the expression of a constitutively active *plo1* allele in the G2 arrested cells as seen with *cut12.s11*. In other words, enhancing Plo1 activity promoted mitosis. The contrasting delay in mitotic commitment in *plo1.ts2* cells suggests that Plo1 is an integral part of the controls that regulate MPF activity during mitotic commitment in fission yeast.

Why are stf mutations not wee?

If *cut12.s11* is activating Plo1 to promote the feedback loop, why does it not make cells divide as soon as Mik1 is destabilized upon completion of S phase and so confer a *wee⁻* phenotype? When *S. pombe* strains are shifted to a poorer or richer nutrient source, there is a transient inhibition of cell division (Fantes and Nurse 1977). This delay in mitotic progression is not seen when *wee1* mutants are shifted between nutrient sources (Fantes and Nurse 1977; Young and Fantes 1987). Two interpretations have been offered for this behavior. Either *wee1* mutants divide below a size threshold, after which they would be able to respond to the nutrient shift, or the nutrient control, which is seen as size control in experimental cultures, acts through Wee1. It is therefore possible that nutrient control stimulates Wee1 activity until cells have attained the critical mass. If this activation can override feedback loop inhibition, which would be a logical hierarchy for the controls, then a constitutively active feedback loop would only be effective in driving mitotic commitment through suppression of Wee1 function once the nutritional/size controls had ceased to stimulate Wee1. Unfortunately, our lack of understanding of the molecular basis for size or nutrient control does not at present permit a direct test of this hypothesis, but it provides a simple explanation as to how the *cut12.s11* could suppress *cdc25* mutants through polo kinase without conferring a *wee⁻* phenotype.

Plo1 association with the SPB

Association of Plo1 with the SPB is clearly complex. We describe three mutants in *plo1* in which the affinity for the interphase SPB of *cut12.s11* cells is reduced below the detection levels of our assays. The inability of Plo1.K69R to bind the interphase SPB of *cut12.s11* suggests that a Plo1-dependent phosphorylation event is required for Plo1 recruitment/maintenance in *cut12.s11*. It is presently unclear whether these phosphorylation events would overlap with, or be independent of, the Fin1-dependent phosphorylation events that are required for Plo1 recruitment to interphase SPBs in *cut12.s11* (Grallert and Hagan 2002). The association of Plo1.ts2 and Plo1.K69R with mitotic SPBs suggests that Plo1 associates with the SPB in more than one manner and probably has multiple docking sites. This would be consistent both with the association of Plo1 with SPB of mitotic cells lacking *cut12* (Mulvihill et al. 1999) and the key role played by Plo1 in regulating the SPB-associated SIN (Tanaka et al. 2001). Given the number of roles emerging for polo kinases in diverse systems, such as the physical association with the microtubule nucleating protein Spc72 (Ho et al. 2002), it is likely that Plo1 will dock with multiple SPB-associated molecules.

Is mitotic commitment controlled from the spindle pole in other eukaryotes?

Microinjection of antibodies against the human polo kinase Plk1 blocks the G2-to-M transition in nontransformed cells, indicating that Plk1 plays a critical role in regulating mitotic commitment in mammalian cells (Lane and Nigg 1996). Both MPF and polo kinase associate with the spindle poles of species as diverse as yeast and man, suggesting that this association may be a key element in the regulation of mitotic commitment (Bailly et al. 1989; Alfa et al. 1990; Golsteyn et al. 1995). The acceleration of MPF activation upon injection of centrosomes into enucleated *Xenopus* eggs would certainly support a role for this organelle in regulating MPF activation (Perez-Mongiovi et al. 2000). Significantly, a recent study has highlighted the importance of an interplay between Plk1 and MPF on the human spindle pole during mitotic commitment (Jackman et al. 2003). Phospho-specific antibodies that recognized residues on cyclin B that have been phosphorylated by either Plk or MPF were used in immunofluorescence studies to identify where MPF was initially activated during mitotic commitment. Both phospho-epitopes appeared first on the centrosome, prior to being seen anywhere else in the cell. Most importantly, phosphorylation of cyclin B by Plk was an early mitotic event that preceded the autophosphorylation of cyclin B by MPF. Thus, it would appear likely that commitment to mitosis in higher eukaryotes is also initiated and potentially regulated by events on the spindle pole.

A role for the spindle pole in regulating mitotic commitment mirrors a growing body of data that describes an essential role for the spindle pole in regulating mitotic exit in yeast, flies, and humans (Bardin and Amon 2001; Khodjakov and Rieder 2001; Piel et al. 2001). The microtubule organizing center is therefore organizing more than just the microtubule array and probably acts as a site upon which diverse regulatory pathways converge to coordinate cell division controls. This would ensure that a decision to progress to the next stage of the cycle is not simply the result of accumulating a threshold level of regulatory proteins at random points in the cell. Rather, the decision is taken by a critical subset of

molecules at the pole before amplification throughout the cell. Regulation from this one critical location makes it easy to ensure full cross-talk between pathways with minimum effort and error.

Materials and methods

Cell culture and strains

The strains used in this study are listed in Table 3. The *plo1.ts* mutants were generated by a marker switch approach that leaves a *ura4⁺* gene 235 bp upstream in the promoter (MacIver et al. 2003). This is indicated by *plo1.x:ura4⁺*, and the wild-type equivalent allele is *plo1⁺:ura4⁺*. Cells were cultured in yeast extract media (YES), appropriately supplemented EMM2 minimal media as described in Moreno et al. (1991). Thiamine-mediated repression of *nmt1⁺* or *nmt41*, HU treatment, and size selection were according to Tanaka et al. (2001).

Molecular genetics

The Quick Change Mutagenesis (Stratagene) approach along with appropriate primers was used to introduce a stop codon and an *Nde*I restriction site at amino acid position 316 of the *plo1⁺* reading frame in pHN180 to generate pPLO316TNdeI. The *Nsi*I–*Bgl*II restriction fragment from pPLO316TNdeI was inserted into p81NPkplo1+ to generate pPK-Nplo316NdeRep81 before subcloning of the 316-amino-acid *Nde*I fragment from this plasmid into pRep81-Pk-N to generate pRep81-PK-Nplo19T. Two-hybrid screening using the restriction fragments

indicated in Figure 1B and C was according to James et al. (1996). Then S124D and T197D were changed into HA-tagged *plo1* in pINT5-41 to make a constitutively active allele of *plo1* referred to as *plo1.S124DT197D* or *plo1.con*. The *Nde*I–*Sma*I fragment of *plo1-con*, lacking the HA tag, and the *Sma*I–*Sma*I *plo1.con* fragment were cloned into pINT6-41 (Drummond and Hagan 1998). The subsequent *plo1.con*-containing *Not*I fragment was integrated into the *leu1* locus of IH415 with *plo1.con* under the control of the *nmt41* promoter.

Microscopy

Plo1 and anti-α-tubulin immunofluorescence procedures were as in Grallert and Hagan (2002) and used anti-Sad1 AP9.2, TAT1, and HN184 (Woods et al. 1989; Hagan and Yanagida 1995; Ohkura et al. 1995). For MPM-2, staining fixation in 3.75% fresh formaldehyde was for 2 min. At least 200 cells were counted for each time point.

Biochemistry

Protein extracts for Western blotting samples were prepared following precipitation with trichloro acetic acid (TCA). A total of 5×10^7 cells were washed and resuspended in stop buffer (Moreno et al. 1991), and TCA was added to a final concentration of 20%. After lysis in a Ribolyser (Hybaid), cells were resuspended in SDS sample buffer. Western blotting with anti-plo1 HN184 sera (Ohkura et al. 1995) and Plo1 kinase assays with HN184 were performed as described previously with the modifications outlined below (Tanaka et al. 2001). For assays following precipitation with MAb336 (Serotec) antibody against the Pk epitope, or to detect coimmunoprecipitation of Plo1 and

Table 3. *Strains used in this study*

Strain number	Genotype	Source
IH136	*cut7.24 leu1.32 h⁻*	Hagan and Yanagida 1990
IH365	*leu1.32 ura4.d18 h⁻*	Lab stock
IH415	*ade6.704 ura4.d18 h⁻*	Lab stock
IH422	*ade6.M210/ade6.M216 mei1.102/mei1⁺*	Lab stock
IH571	*cut12.NEGFP leu1.32 ura4.d18 h⁻*	Bridge et al. 1998
IH666	*cut12.s11 leu1.32 h⁺*	Hudson et al. 1990
IH740	*cdc25.22 cut12.s11 leu1.32 ura4.d18 h⁺*	Lab stock
IH741	*cdc25.22 cut12.s11 ura4.d18 h⁻*	Bridge et al. 1998
IH470	*cdc2.33 ade6.704 leu1.32 ura4.d18 h⁻*	This study
IH1899	*cdc2.33 ade12.s11 leu1.32 ura4.d18 h⁻*	This study
IH754	*cut12.1 ade6.704 ura4.d18 h⁻*	Bridge et al. 1998
IH1625	*plo1.ts2:ura4⁺ leu1.32 ura4.d18 h⁻*	This study
IH1636	*plo1.ts19:ura4⁺ leu1.32 ura4.d18 ade6.M210 h⁻*	This study
IH1963	*cdc25.22 leu1.32 ura4.d18 h⁻*	Fantes 1981
IH2122	*plo1.ts19:ura4⁺ cdc25.22 cut12.s11 ura4.d18 h⁻*	This study
IH2156	*plo1.ts2:ura4⁺ cdc25.22 cut12.s11 ura4.d18*	This study
IH2311	*plo1.ts2:ura4⁺ cut12.s11 ura4.d18*	This study
IH2444	*ura4.d18 leu1.32 h⁻ pREP81 PK-N empty vector*	This study
IH2230	*ura4.d18 leu1.32 h⁻ pREP81-PK-N-plo19T*	This study
IH2445	*ura4.d18 leu1.32 h⁻ pREP81 PK-N plo1⁺*	This study
IH2514	*plo1.ts19:ura4⁺ leu1::plo1⁺ ura4.d18*	This study
IH2515	*plo1.ts2:ura4⁺ leu1::plo1⁺ ura4.d18*	This study
IH2550	*cut12.s11 leu1.32 h⁺ pREP81-Pk-N-plo1⁺*	This study
IH2551	*cut12.s11 leu1.32 h⁺ pREP81-PK-N-ploK69R*	This study
IH2613	*plo1⁺:ura4⁺ cdc25.22 cut12.s11 ura4.d18 leu1.32*	This study
IH2893	*leu1::plo1.S124DT197D(nmt41):ura4⁺ leu1.32 ade6.704 ura4.d18*	This study
IH2899	*leu1::plo1.S124DT197D(nmt41):ura4⁺ cdc25.22 ura4.d18 leu1.32*	This study
IH2928	*leu1::plo1.S124DT197D(nmt41):ura4⁺ cdc2.33 ura4.d18 ade6*	This study

Cut12, Dynabeads (Dynal) rather than Sepharose beads were used. The antibody was covalently coupled to beads according to the manufacturer's instructions. Following the immunoprecipitation, the immunocomplex was washed once in WB1 (50 mM HEPES at pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100), twice in WB2 (50 mM HEPES at pH 7.5, 1 mM EDTA), and resuspended in CK buffer (Tanaka et al. 2001) before being run on a gel or used in a kinase assay. Cut12 was detected with antibodies as described in Bridge et al. (1998). Histone H1 kinase assays were performed according to the protocol of Creanor and Mitchison (1994).

Acknowledgments

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