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Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage

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In yeast, anaphase entry depends on Pds1 proteolysis, while chromosome re-duplication in the subsequent S-phase involves degradation of mitotic cyclins such as Clb2. Sequential proteolysis of Pds1 and mitotic cyclins is mediated by the anaphase-promoting complex (APC). Lagging chromosomes or spindle damage are detected by surveillance mechanisms (checkpoints) which block anaphase onset, cytokinesis and DNA re-replication. Until now, the *MAD* and *BUB* genes implicated in this regulation were thought to function in a single pathway that blocks APC activity. We show that spindle damage blocks sister chromatid separation solely by inhibiting APC^{Cdc20}-dependent Pds1 proteolysis and that this process requires Mad2. Blocking APC^{Cdh1}-mediated Clb2 proteolysis and chromosome re-duplication does not require Mad2 but a different protein, Bub2. Our data imply that Mad1, Mad2, Mad3 and Bub1 regulate APC^{Cdc20}, whereas Bub2 regulates APC^{Cdh1}.

Keywords: Bub2/cell cycle arrest/Mad2/spindle checkpoint

Introduction

The duplication of chromosomes, the separation of sister chromatids and their segregation to opposite poles of the cell prior to cytokinesis are essential features of the eukaryotic cell cycle. Cells possess regulatory mechanisms that initiate these events in the correct order and that render cell cycle progression dependent on successful completion of preceding events. These mechanisms ensure that chromosome duplication occurs only once per cell cycle, and that it alternates with the processes of sister chromatid separation and cytokinesis. Furthermore, these mechanisms contribute to the high fidelity of chromosome transmission by providing additional time to repair DNA damage or to complete assembly of the mitotic apparatus.

The chromosome cycle starts with DNA replication, whose initiation takes place in two steps. Assembly of replication-competent complexes at future origins occurs upon inactivation of mitotic cyclin-dependent kinases (CDKs) as cells exit from mitosis, whereas the emergence of replication forks from these origins only occurs upon activation of S phase-promoting CDKs in late G_1 (Tanaka and Nasmyth, 1998). Chromosome duplication produces a pair of sister chromatids bound together by a multi-subunit Cohesin complex (Guacci *et al*., 1997; Michaelis *et al*., 1997; Losada *et al*., 1998) which forms joints between sisters during the process of replication (Uhlmann and Nasmyth, 1998). Cohesin holds sisters together throughout G_2 and subsequently during their alignment on the mitotic spindle when it opposes the splitting force exerted by the spindle. In yeast, the eventual separation of sister chromatids during anaphase is thought to be triggered by dissolution of the linkage between sisters mediated by Cohesin. Scc1, an essential subunit of Cohesin, disappears from chromosomes at the point of their separation (Michaelis *et al*., 1997). Furthermore, this process depends on a sister separating protein (Separin) called Esp1, which is essential for sister chromatid separation but not for other anaphase events (Ciosk *et al*., 1998).

Sister chromatid separation and chromosome re-duplication both depend on proteolysis of regulatory molecules. In budding yeast, sister chromatid separation depends on proteolysis of Pds1 (Cohen-Fix *et al*., 1996), which binds to and inhibits Esp1 (Ciosk *et al*., 1998). Assembly of replication-competent complexes on origins, the first step in chromosome re-duplication, involves proteolysis of B-type cyclins such as Clb2, Clb3 and Clb5 (Nasmyth, 1996). Degradation of all these proteins depends on a ubiquitin protein ligase called the anaphase-promoting complex (APC) or cyclosome (Irniger *et al*., 1995; King *et al*., 1995; Sudakin *et al*., 1995). However, proteolysis of different APC substrates commences at different stages of mitosis and depends on different activator proteins. Pds1 destruction occurs shortly before the onset of anaphase and depends on Cdc20 (Visintin *et al*., 1997), whereas Clb2 destruction only occurs later during anaphase and depends on a different, but related activator called Cdh1 or Hct1 (Schwab *et al*., 1997; Visintin *et al*., 1997).

To avoid missegregation of chromosomes, anaphase must only be initiated after sister chromatids of each duplicated chromosome have attached to opposite poles of the mitotic spindle. Microtubules emanating from opposing spindle poles attach to a specialized structure on each sister chromatid called the kinetochore. Microtubules are thought to 'find' kinetochores by a 'search and capture' mechanism which cannot be completed simultaneously for all chromosomes (Hayden *et al*., 1990; Merdes and De Mey, 1990). Cells therefore possess regulatory mechanisms that delay sister chromatid separation, cytokinesis and chromosome re-duplication until the last chromosome has achieved bipolar attachment. Mechanisms required to enforce the dependency of cell cycle progression on the completion of previous steps have been termed surveillance mechanisms or checkpoints (Hartwell and Weinert, 1989). Similar if not identical mechanisms block cell cycle progression after the artificial destruction of the spindle by microtubule-depolymerizing

drugs such as nocodazole or benomyl. Neither Pds1 nor Clb2 are degraded when cells are treated with nocodazole, presumably because mitotic surveillance mechanisms inhibit the APC.

The isolation of mutants that die rapidly when treated with drugs such as nocodazole has identified several genes thought to be involved in mitotic surveillance: *MAD1*, *MAD2*, *MAD3*, *BUB1*, *BUB2*, *BUB3*, *PDS1* and *MPS1* (Hoyt *et al*., 1991; Li and Murray, 1991; Weiss and Winey, 1996; Yamamoto *et al*., 1996). These mutants have been shown to be defective in halting various aspects of the cell cycle in the absence of mitotic spindles, at least after long periods in the presence of nocodazole. *MAD1*, *MAD2*, *MAD3*, *BUB1* and *BUB3* have homologues in multicellular eukaryotes, including humans (Chen *et al*., 1996, 1998; Li and Benezra, 1996; Pangilinan *et al*., 1997; Jin *et al*., 1998; Taylor *et al*., 1998). Interestingly, vertebrate homologues of Mad2, Bub1 and Bub3 were found to associate with kinetochores prior to chromosome alignment on the metaphase plate (Chen *et al*., 1996; Li and Benezra, 1996; Taylor and McKeon, 1997; Taylor *et al*., 1998). The immunolocalization to kinetochores is lost after chromosomes become properly attached to the mitotic spindle at metaphase, suggesting that these proteins might monitor the completeness of the spindle–kinetochore attachment. Mad proteins were also found to be directly involved in blocking cell cycle progression. Mad2 and Mad3 bind to Cdc20 and, furthermore, Cdc20 mutants defective in Mad2 association allow cells to escape from the mitotic arrest (Hwang *et al*., 1998). These yeast data, together with data from the study of human Mad2 (Fang *et al*., 1998), suggest that Mad proteins block sister chromatid separation by inhibiting the activity of APC^{Cdc20}. It is unclear, however, whether the same proteins also inhibit APC^{Cdh1} and, moreover, how Bub proteins contribute to cell cycle arrest.

It is currently thought that sister chromatid separation might be triggered by Pds1 proteolysis. Expression of a non-degradable version of Pds1 does indeed block entry into anaphase (Cohen-Fix *et al*., 1996). However, a dependence of sister separation on Pds1 destruction does not necessarily imply that Pds1 destruction is therefore rate limiting for sister separation in wild-type cells. Indeed, deletion of *PDS1* is not lethal, at least when cells are grown at low temperatures. Other mechanisms clearly exist that are capable of regulating the metaphase to anaphase transition in yeast.

The main task of biology is to determine how wild-type cells function. Might Pds1 destruction be the rate-limiting step in wild-type cells? To address this question, we compared the kinetics of sister separation in wild-type and *pds1* mutants. Somewhat to our surprise, we found little or no difference. This suggests that a Pds1-independent mechanism contributes to the timing of anaphase onset in wild-type yeast cells. What then is Pds1's main role in the yeast cell cycle? Why do yeast cells subjugate the segregation of their chromosomes by the need to degrade Pds1? It appears that cell cycle arrest in response to DNA or spindle damage depends on Pds1; that is, cells arrest nuclear division by blocking Pds1 destruction (Yamamoto *et al*., 1996; Cohen-Fix and Koshland, 1997). We therefore investigated further the mechanism by which Mad proteins and Pds1 block sister chromatid separation when spindles are severely damaged by nocodazole. It has already been shown that sister separation occurs in ~50% of *pds1* mutant cells after 5 h incubation in nocodazole (Yamamoto *et al*., 1996). This observation does not, however, exclude the possibility that *pds1* mutant cells are capable of delaying sister separation for several hours (i.e. for up to one generation time). We therefore measured the response of *pds1* and *mad* mutants to nocodazole using cells synchronized by centrifugal elutriation. Our data suggest that Mad proteins block sister separation solely by inhibiting Pds1 proteolysis and that this alone is responsible for inhibiting Esp1. They therefore confirm what till now had merely been a reasonable working hypothesis. However, contrary to previous conclusions, Mad proteins are not required to block Clb2 degradation and chromosome re-replication. These processes are regulated by Bub2, which has little or no direct role in the regulation of Pds1 proteolysis. Our data suggest that proteolysis mediated by APC^{Cdc20} and APC^{Cdh1} is regulated by different proteins. Mad proteins are largely concerned with blocking sister chromatid separation, whereas Bub2 is concerned with blocking exit from mitosis, cytokinesis and chromosome re-duplication.

Results

MAD2 but not BUB2 is essential for blocking Pds1 degradation in cells treated with nocodazole

Pds1 degradation, mediated by APC^{Cdc20}, is an essential pre-condition for sister chromatid separation (Cohen-Fix *et al*., 1996). Mad2 binds APC^{Cdc20} and inactivates it upon nocodazole treatment (Fang *et al*., 1998; Hwang *et al*., 1998). To address whether spindle checkpoint proteins arrest the cell cycle by blocking Pds1 proteolysis, we compared the kinetics of Pds1 degradation in wild-type, *mad2* and *bub2* deletion strains as small G_1 cells were incubated at 25°C in the presence of nocodazole. The drug completely blocked Pds1 degradation in wild-type cells (Figure 1A) but had no effect in *mad2* mutant cells; Pds1 disappeared from nuclei with kinetics similar if not identical to those observed in wild-type cells grown in the absence of nocodazole (Figure 1B). In contrast, *BUB2* was not required to block Pds1 degradation (Figure 1C), indicating that Bub2 has a different role than Mad2 in the spindle checkpoint arrest. We conclude that in cells treated with nocodazole, Mad2 but not Bub2 is essential for blocking degradation of Pds1.

Mad2 and Pds1 are essential for blocking dissociation of Scc1 from chromosomes in cells treated with nocodazole

We next asked whether Mad2 is essential for regulating sister chromatid separation when spindles are damaged. Minshull *et al*. (1996) have already reported that 50% of *mad2* mutant cells fail to hold sister chromatids together when incubated in nocodazole. By visualizing the chromosomes using a *tet* repressor–green fluorescent protein fusion (*tet*R–GFP) which binds to an array of *tet* operators integrated near the centromere of chromosome V (Michaelis *et al*., 1997), we were not able to detect more than 30% cells with separated GFP 'dots' and only after a long incubation in nocodazole. To avoid the risk of underestimating the efficiency of sister chromatid separation by measuring the physical separation of sisters in

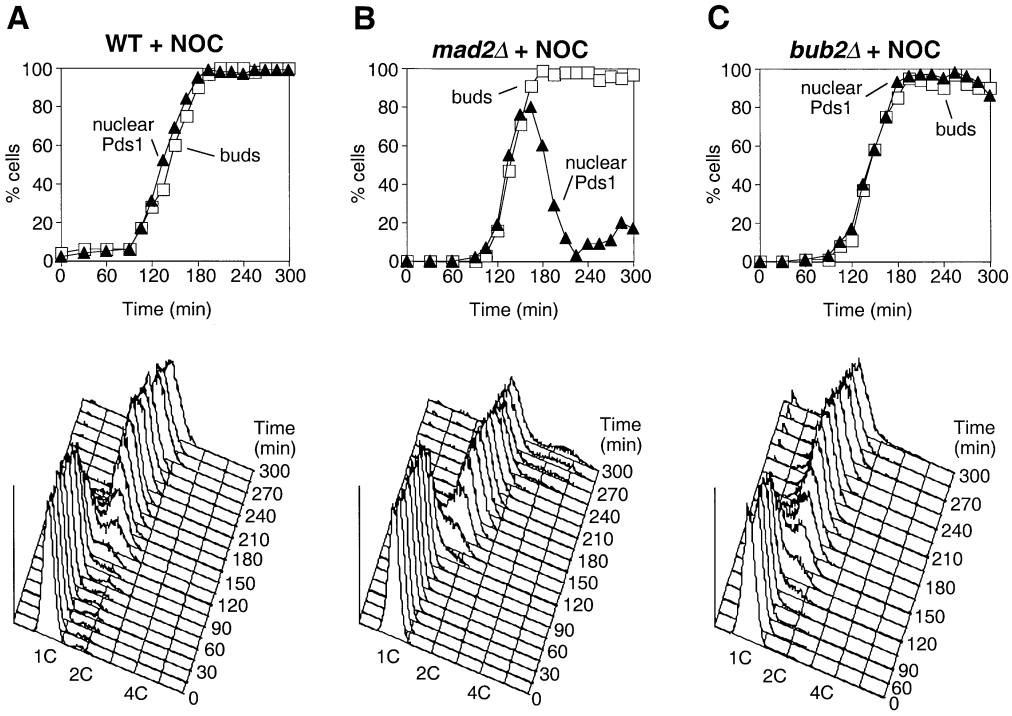


Fig. 1. Mad2, but not Bub2 is essential for Pds1 stabilization in nocodazole-treated cells. G_1 cells of wild-type (K6803) (**A**), *mad2Δ* (K7292) (**B**) and *bub2Δ* (K7145) (**C**) strains containing *PDS1-myc18* were isolated by centrifugal elutriation and incubated at 25°C in the presence of nocodazole. The percentage of budded cells (□) and cells positive for nuclear Pds1-Myc18 staining (▲), as determined by indirect immunofluorescence, were scored in samples taken at the times indicated. Bottom panels, cellular DNA content as measured by flow cytometry.

cells that possess no means of pulling them apart, we analysed the chromosomal association of an epitope-tagged variant of Scc1 (Scc1-myc18) using 'chromosome spreads'. Scc1 is essential for establishment and maintenance of sister chromatid cohesion and suddenly disappears from chromosomes at the time of their separation (Michaelis *et al*., 1997). Recent data suggest that the disappearance of Scc1 from chromosomes is synonymous with loss of sister chromatid cohesion (F.Uhlmann and K.Nasmyth, personal communication). Scc1's association with chromosomes was analysed as G_1 cells of a *mad2* deletion strain progressed through the cell cycle at 25°C, in both the absence and presence of nocodazole. The drug completely blocks Scc1 dissociation in wild-type cells (Uhlmann and Nasmyth, 1998), but it had no effect on the kinetics of this process in *mad2* mutant cells (Figure 2A and B).

To test whether Mad2's role in inhibiting Scc1 dissociation from chromosomes is limited to blocking Pds1 proteolysis, we analysed Scc1's disappearance from chromatin as G_1 cells from a *pds1* deletion strain progressed through the cell cycle at 25°C, and we observed that nocodazole failed to delay Scc1's dissociation from chromosomes (Figure 2C and D). We conclude that both *MAD2* and *PDS1* are essential for blocking Scc1's disappearance from chromosomes when spindles are damaged by nocodazole. Our data suggest that Mad2 blocks sister chromatid separation exclusively by blocking proteolysis of Pds1, which binds to and inhibits the sister separating protein Esp1. Despite being completely defective in arresting sister chromatid separation, *mad2* mutant cells were capable of preventing cytokinesis and DNA re-replication (Figure 2B). This finding demonstrates that the Mad2-dependent surveillance mechanism inhibits a subset of the events that are blocked by damaging spindles. Mad2's role in arresting the cell cycle is therefore more specific than hitherto suspected (Minshull *et al*., 1996).

Cell cycle arrest due to over-expression of MPS1 depends on PDS1

Our analysis of Scc1 circumvented the problems associated with measuring sister separation accurately in cells that possess no spindle apparatus. We wished nevertheless to assess more directly Pds1's role in blocking sister separation. The activation of the spindle checkpoint without inducing spindle damage by over-production of the Mps1 protein-kinase provided a suitable opportunity. Over-expression of *MPS1* causes a *MAD*/*BUB*-dependent cell cycle arrest in cells with functional spindles (Hardwick *et al*., 1996). If Pds1 were the sole means by which Mad/Bub proteins block sister separation, then cell cycle arrest mediated by *GAL-MPS1* should be dependent on *PDS1*. To test this, small G_1 cells of wild-type and *pds1* mutant strains over-expressing *MPS1* from the *GAL* promoter were isolated by centrifugal elutriation and released into galactose medium. Mps1 over-production completely blocked anaphase entry in wild-type cells (Figure 3A), but it had no effect on nuclear division and the formation of anaphase spindles in *pds1* mutant cells (Figure 3B). These data confirm that Pds1 is essential to block entry into anaphase upon activation of the spindle checkpoint.

Pds1 is not required to prevent precocious sister chromatid separation during a normal cell cycle

The fact that Pds1 is destroyed shortly before sisters separate (Michaelis *et al*., 1997) suggests that Pds1

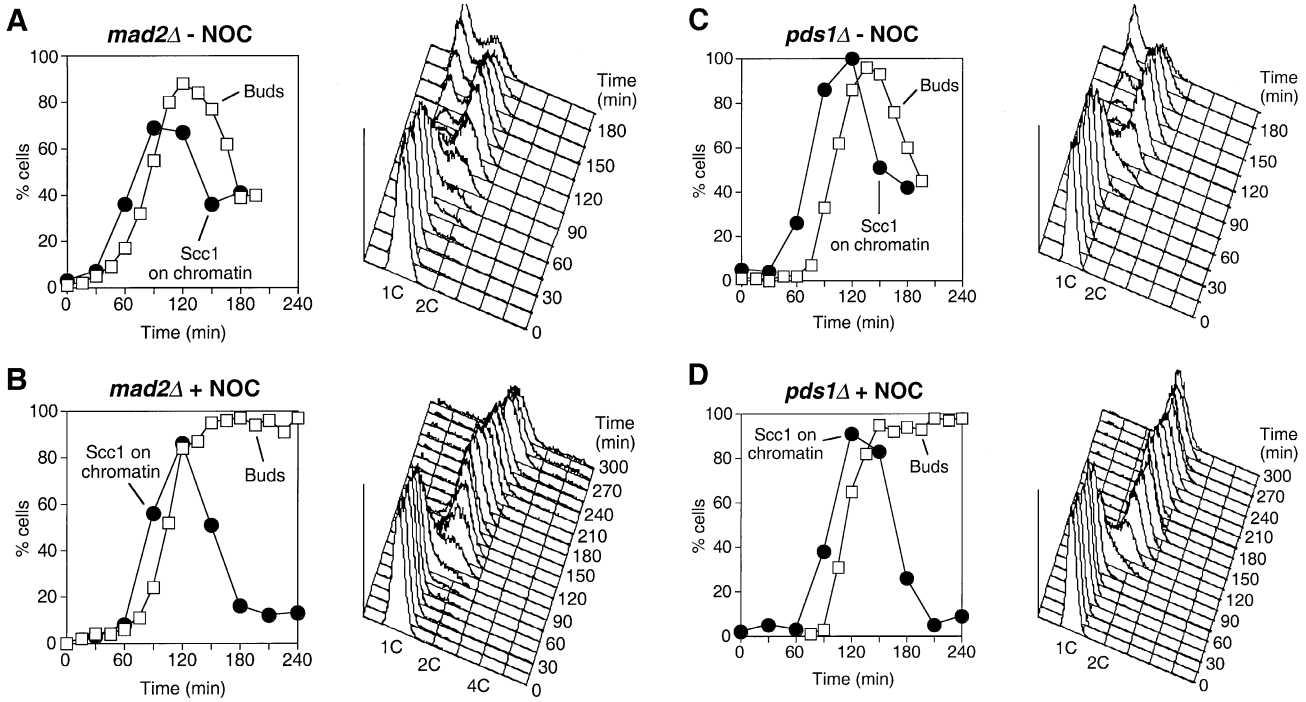


Fig. 2. Mad2 and Pds1 are essential to block anaphase onset upon spindle checkpoint activation. (A and B) G_1 cells of a *mad2Δ SCC1-myc18* strain (K7408) were incubated in the absence (**A**) or presence (**B**) of nocodazole. The fraction of cells containing Scc1-Myc18 bound to chromatin was determined by chromosome spreading (●). (C and D) Scc1 association with chromatin was also analysed in a *pds1Δ SCC1-myc18* strain (K7404) as G_1 cells progressed through the cell cycle in the absence (**C**) or presence (**D**) of nocodazole. Panels on the right show cellular DNA content.

proteolysis might actually trigger the metaphase to anaphase transition, in which case *pds1* mutants should separate sister chromatids precociously. To test this, we compared the kinetics with which sequences adjacent to centromere V (CenV) separated in wild-type and *pds1* deletion strains as small G_1 cells isolated by centrifugal elutriation were incubated at 25°C. CenV sequences, visualized using the *tet*R–GFP/*tet* operator system, separated with similar kinetics ~60 min after bud emergence in both strains (Figure 4A and B). This suggests that sister separation is controlled by a second mechanism which does not involve Pds1. This Pds1-independent mechanism does not depend on *MAD2*, because sister separation occurred with similar kinetics in *mad2* single mutants (Figure 4C) and in *pds1mad2* double mutants (Figure 4D).

Neither Mad2 nor Pds1 are required to delay Clb2 proteolysis in nocodazole-arrested cells

Cytokinesis and DNA re-replication are known to depend on inactivation of Cdk1 kinases (Surana *et al*., 1993; Dahmann *et al*., 1995). The failure of *mad2* mutant cells to undergo cytokinesis and re-replication therefore suggests that these cells maintain Cdk1 activity. To test this notion, we analysed the levels of Clb2 and Clb3 following incubation of small G_1 cells of wild-type and *mad2* mutants in the absence (Figure 5A and C) and in the presence of nocodazole (Figure 5B and D). The drug had no influence on the appearance of either protein. Clb3 accumulated during S phase and Clb2 accumulated ~15 min later. In the absence of nocodazole, Clb3 and Clb2 declined 65 and 80 min, respectively, after entry into S-phase. Clb2-associated Cdk1 kinase activity had a similar profile. In wild-type cells, nocodazole, as

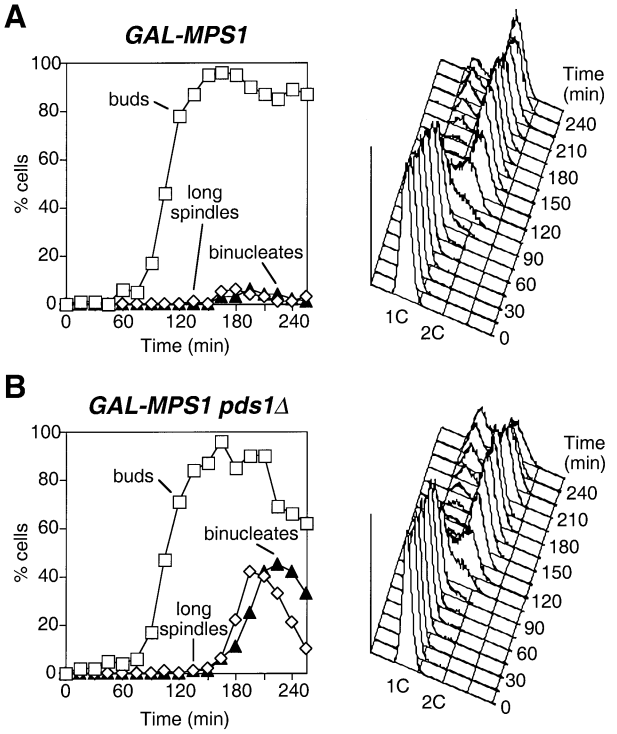


Fig. 3. *PDS1* deletion causes cells over-expressing *MPS1* to undergo anaphase. Small G_1 cells of a *GAL-MPS1* (K7401) (**A**) and a *GAL-MPS1 pds1Δ* strain (K7418) (**B**) were released into galactose medium. The percentage of budded cells (□), cells containing long spindles (◇) and cells with separated DNA masses (binucleate, ▲) were scored. Right panels indicate cellular DNA content.

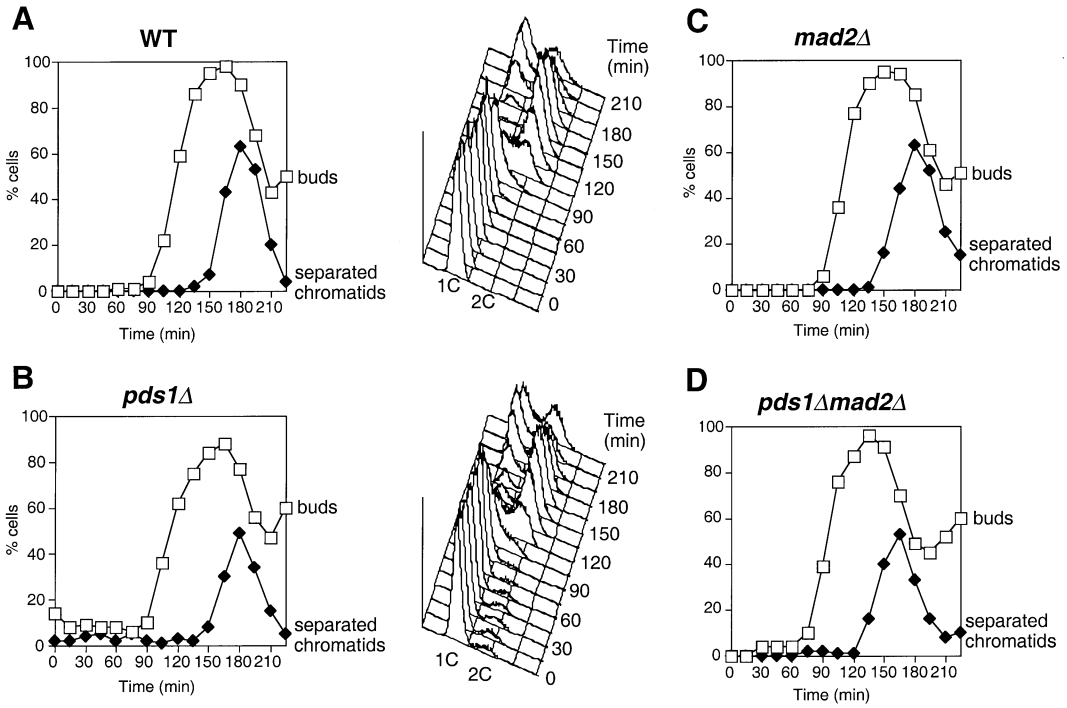


Fig. 4. Sister chromatid separation occurs with wild-type kinetics in the absence of Pds1, Mad2 or both proteins. G_1 cells of wild-type (K6745) (**A**), *pds1Δ* (K6885) (**B**), *mad2Δ* (K7292) (**C**) and *pds1Δmad2Δ* (K7297) (**D**) strains containing CenV-GFP were isolated by centrifugal elutriation and incubated at 25°C. The percentage of budded cells (□) and cells with separated sister chromatids at CenV (two GFP 'dots', ◆) were determined. (A and B) On the right, cellular DNA content.

expected, blocked the degradation of both Clb2 and Clb3 (Figure 5B). Clb3 protein levels also dropped in *mad2* mutants incubated in nocodazole, 80 min after cells initiated S-phase, but Clb2 levels and Clb2-associated kinase activity continued to accumulate for 1 h and only then started to decline, albeit very slowly (Figure 5D). We conclude that Mad2 is required to block degradation of Pds1 and, to a certain extent, also that of Clb3. However, Mad2 is not required to delay Clb2 proteolysis or inactivation of its associated Cdk1 kinase. A Mad2-independent mechanism must largely be responsible for blocking Clb2–Cdk1 kinase inactivation, cytokinesis and DNA re-replication.

We also measured the levels of Clb2 and Clb3 following incubation of G_1 *pds1* mutant cells in nocodazole. We found that neither protein was degraded and that cells neither underwent cytokinesis nor re-replicated their chromosomes (Figure 5E). The previous observation that histone H1 kinase remains high several hours after incubation of *pds1* mutants with nocodazole (Yamamoto *et al*., 1996) is consistent with our measurement of Clb2 and Clb3 levels.

Our finding that ⩾95% of *mad2* mutant cells maintain high levels of Clb2–Cdk1 kinase and fail to re-replicate their genomes in the presence of nocodazole is inconsistent with an earlier conclusion that Mad2 is necessary to block inactivation of Clb2–Cdk1 (Minshull *et al*., 1996). In the latter study, cells were arrested with α-factor and released into medium containing nocodazole. We note, however, that Clb2–Cdk1 kinase did not drop more than 2-fold from its peak level in *mad2* mutants and then persisted despite re-addition of α-factor. If cells were completely defective in blocking exit from mitosis they would be expected to arrest in G_1 and the Clb2–Cdk1 kinase should disappear. To address whether the method of synchronization might be responsible for our different results, we analysed the DNA content of wild-type and *mad2* mutant cells after their release from α-factor. This confirmed that *mad2* mutants delay cell cycle progression by at least one generation time even when synchronized by pheromone release: 3 h after release in the absence of nocodazole, wild-type cells underwent a second round of DNA replication, while the majority of *mad2* mutant cells released in the presence of nocodazole are still arrested with 2C DNA content (Figure 5F). Upon longer incubation in nocodazole, a fraction of *mad2* mutant cells indeed re-replicate their DNA which may account for the drop of Cdk1 activity observed by Minshull *et al*. (1996).

Bub2 and Pds1 can block Clb2 degradation independently

As shown in Figure 1C, *bub2* mutants treated with nocodazole block not only Pds1 proteolysis, but also cytokinesis and DNA re-replication, consistent with their ability to block Clb2 and Clb3 proteolysis and to maintain high levels of Clb2-associated–Cdc28 kinase activity (Figure 6A). One possibility is that Pds1, which persists in *bub2* mutants, in addition to inhibiting Esp1, blocks Clb2 proteolysis by a mechanism that is independent of Bub2. This hypothesis is consistent with the observation that expression of a non-degradable version of Pds1 not only blocks sister chromatid separation but also Clb2 proteolysis (A.Toth, personal communication). To test this, Clb2 and Clb3 protein levels were measured as small G_1 cells of a *bub2pds1* double mutant strain were incubated in medium containing nocodazole at 25°C (Figure 6B). Both proteins were degraded and furthermore, the double mutant cells re-replicated their chromosomes, albeit more

slowly than wild-type cells incubated in the absence of nocodazole (compare Figures 6B and 4A). These data show that the block to Clb2 degradation in *bub2* mutants is dependent on Pds1 and that the same block in *pds1* mutants is dependent on Bub2. Thus, nocodazole blocks mitotic cyclin degradation and re-replication by two independent mechanisms: a Mad2-dependent pathway blocks Pds1 degradation and thereby that of Clb2, whereas a Bub2-dependent pathway blocks Clb2 degradation in a Pds1-independent manner.

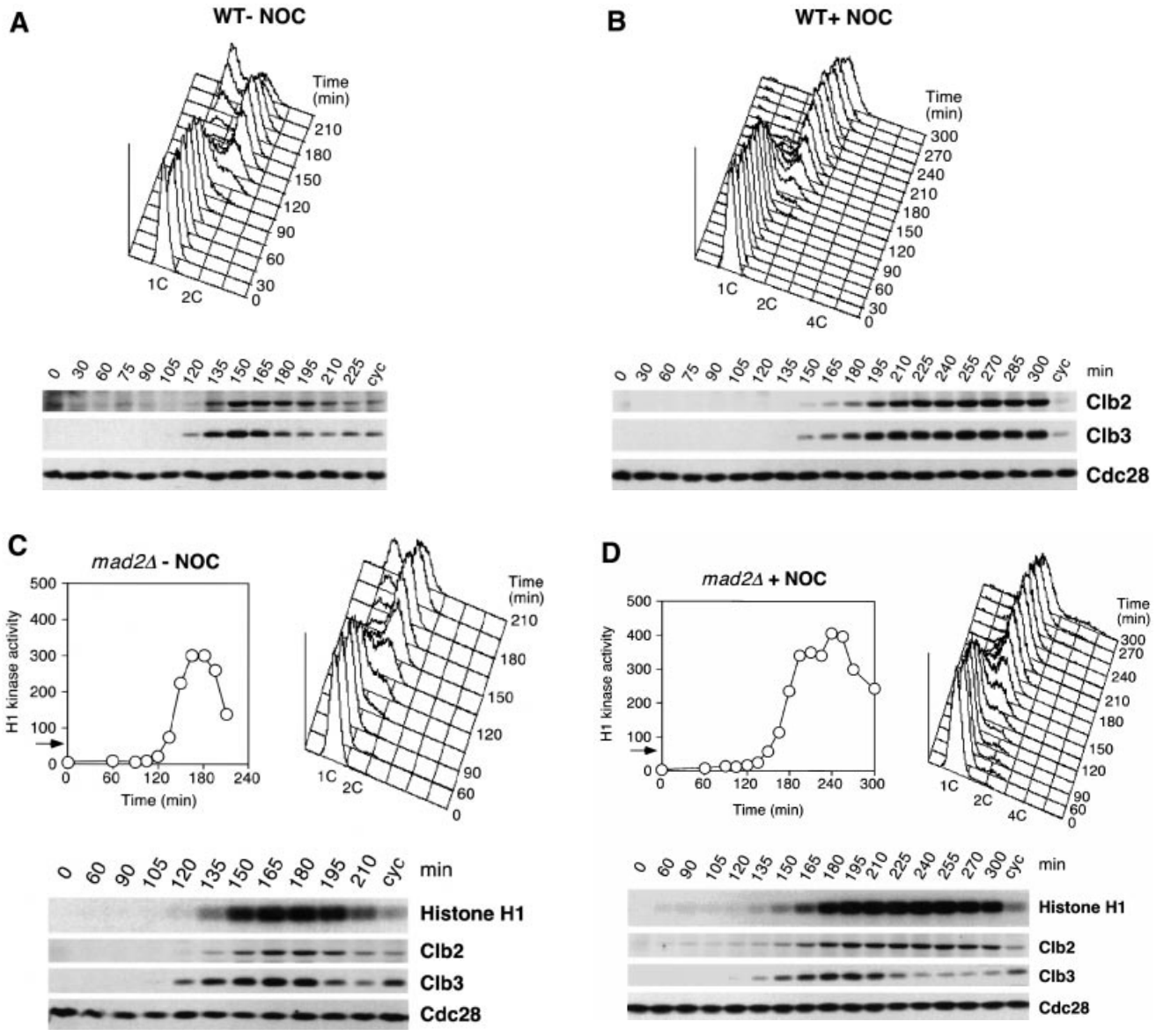
bub2mad2 double mutants re-replicate their DNA with wild-type kinetics in nocodazole

Our data suggest that at least two different pathways respond to microtubule depolymerization by nocodazole: a Mad2-dependent pathway blocks Pds1 degradation and sister chromatid separation, whereas a Bub2-dependent pathway blocks Clb2 degradation and re-replication. To test whether inactivation of both pathways would completely abrogate cell cycle arrest due to nocodazole, we analysed cell cycle progression of *bub2mad2* double mutant cells in the presence of nocodazole (Figure 6C). We observed, remarkably, that these cells re-replicated their genomes with kinetics that were very similar to wild-type cells incubated in the absence of nocodazole. The double mutant cells also degraded Clb2 and Clb3 (with kinetics that were similar to wild-type cells incubated in the absence of nocodazole) and even underwent cytokinesis, producing cells with little or no DNA and cells with a 4C DNA content.

DNA re-replication in *bub2mad2* double mutant cells was more efficient than in *bub2pds1* double mutant cells (compare Figure 6B and C), which suggests that Mad2 blocks proteolysis of proteins other than Pds1, whose persistence in *bub2pds1* cells interferes with re-replication. A candidate for such a protein is Clb3, which is degraded in *mad2* mutant cells treated with nocodazole (Figure 5D), but not in *pds1* mutant cells in nocodazole (Figure 5E).

Bub2 functions in a pathway that is different to that of other Mad and Bub proteins

The efficient re-replication in the presence of nocodazole of *bub2mad2* double mutants but not either single mutant suggests that Mad2 and Bub2 function in different regu-



latory pathways. To assign other *MAD* and *BUB* genes to these two different pathways, we analysed the cellular DNA contents of various *bubmad* double mutants. We first established that it is possible to distinguish the behaviour of wild-type, *mad* and *bub* single mutants, and *bub2mad2* double mutants by following the cellular DNA content of asynchronous cultures incubated for 3 h in the presence of nocodazole: wild-type and single mutant cells arrested with a 2C DNA content, whereas the *bub2mad2* double mutant cells accumulated a 4C DNA content (Figure 7). Consistent with previously published data (Schott and Hoyt, 1998), a fraction of *mad2*, *mad3* and *bub2* single mutant cells re-replicated their DNA after longer incubation in nocodazole. *bub1mad1*, *bub1mad2*, *mad1mad2* and *mad2mad3* double mutants all resembled wild-type, but *bub2mad3*, *bub2mad1* and *bub1bub2* double mutants underwent re-replication (Figure 7). These data suggest that Mad1, Mad2, Mad3 and Bub1 all belong to the same regulatory pathway, which is distinct from that of Bub2.

The Bub2-dependent pathway is functional in cells carrying a Mad2-resistant allele of CDC20

Our conclusion that Mad2 and Bub2 can independently block cell cycle progression upon spindle checkpoint activation is apparently inconsistent with the existence of *CDC20* alleles defective in blocking re-budding and DNA re-replication (Hwang *et al*., 1998; Schott and Hoyt, 1998). To test whether *CDC20-107* mutant cells are completely defective in the spindle checkpoint response we compared cellular DNA contents of *CDC20-107* single mutants and *CDC20-107 bub2* double mutants as elutriated G_1 cells progressed through the cell cycle in the presence of nocodazole. Few if any *CDC20-107* mutant cells re-replicated their DNA within a period in excess of one generation time, similar to *mad2* deletion mutants (compare Figures 8A and 1B), while *CDC20-107 bub2* double mutants efficiently re-replicated their DNA resembling *bub2mad2* mutants (compare Figures 8B and 6C). A crucial difference between our studies and previous ones is the time course over which cells have been observed: a fraction of the elutriated *CDC20-107* single mutant cells also re-replicated after extended incubation in nocodazole (data not shown). These data confirm that unlike Mad2, Bub2 functions independently of Cdc20.

DNA re-replication in bub2mad2 double mutant cells treated with nocodazole depends on Cdc14

To maintain high levels of Cdk1 activity, Bub2 must regulate proteolysis of both Clb2 and the Cdk inhibitor Sic1. Therefore, it is unlikely that Bub2 inhibits the APC directly as does Mad2, but rather a protein that regulates both processes. A good candidate is the Cdc14 phosphatase, which is essential for inactivation of Clb2–Cdk1 at the end of mitosis (Fitzpatrick *et al*., 1998). Cdc14 has been shown to de-phosphorylate Sic1 and Swi5, both of which would contribute to Sic1 accumulation (Visintin *et al*., 1998). Cdc14 also de-phosphorylates Cdh1 (Jaspersen *et al.*, 1999), which would permit Cdh1 to bind to the APC and thereby activate Clb2 proteolysis (Zachariae *et al*., 1998). If this hypothesis is correct, then the re-replication of *bub2mad2* double mutants in the presence of nocodazole should be dependent on Cdc14. To test this, nocodazole was added to wild-type, *bub2mad2*

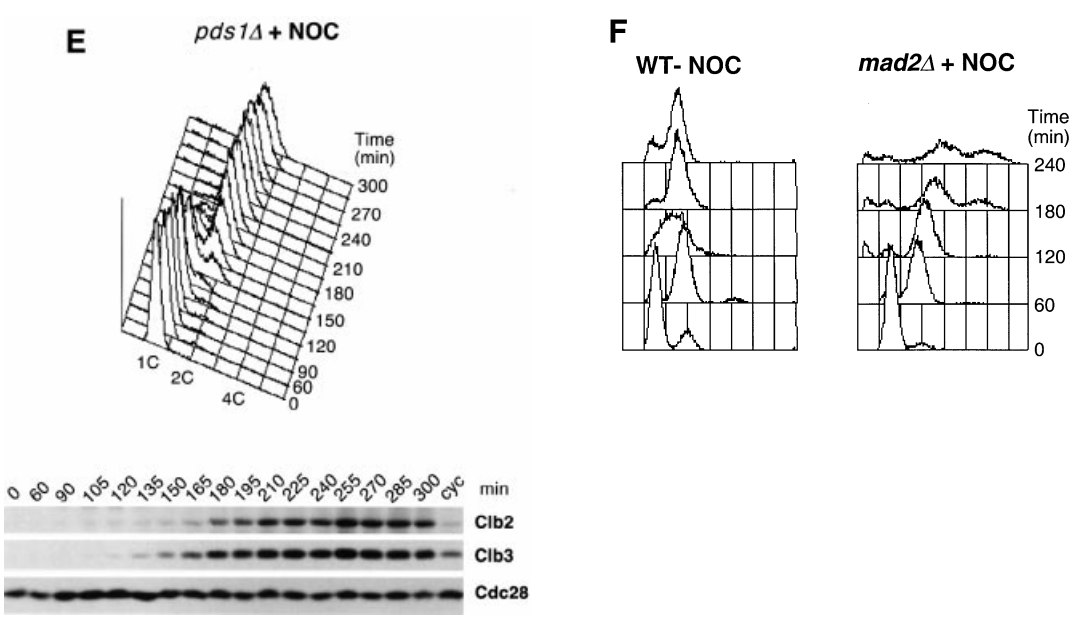


Fig. 5. *mad2Δ* and *pds1Δ* mutants block Clb2–Cdk1 kinase inactivation and DNA re-replication in the presence of nocodazole. (A and B) G_1 cells of a wild-type strain (K699) were isolated by centrifugal elutriation and incubated in the absence (**A**) or presence (**B**) of nocodazole. Protein levels of Clb2 and Clb3 were analysed in samples withdrawn at the time points indicated. Cdc28 was detected as a loading control. Upper panels indicate the cellular DNA content. (C and D) Protein levels of Clb2 and Clb3 and the activity of the Clb2-associated histone H1 kinase as elutriated G_1 cells of a *mad2Δ* strain (K6599) progressed through the cell cycle in the absence (**C**) or presence (**D**) of nocodazole. The amount of phosphorylated histone H1 was quantified by phosphoimaging (○). The activities measured in the two cultures were normalized using the sample from cycling cells (indicated with an arrow in the graphs). Measurements of cellular DNA content are shown on the right. (**E**) Protein levels of Clb2 and Clb3 in *pds1* mutants after incubation of elutriated G_1 cells in nocodazole. Upper panel shows the cellular DNA content. (**F**) Comparison between the cellular DNA contents of α-factor arrested wild-type cells released in the absence of nocodazole and that of *mad2* mutant cells released in the presence of nocodazole.

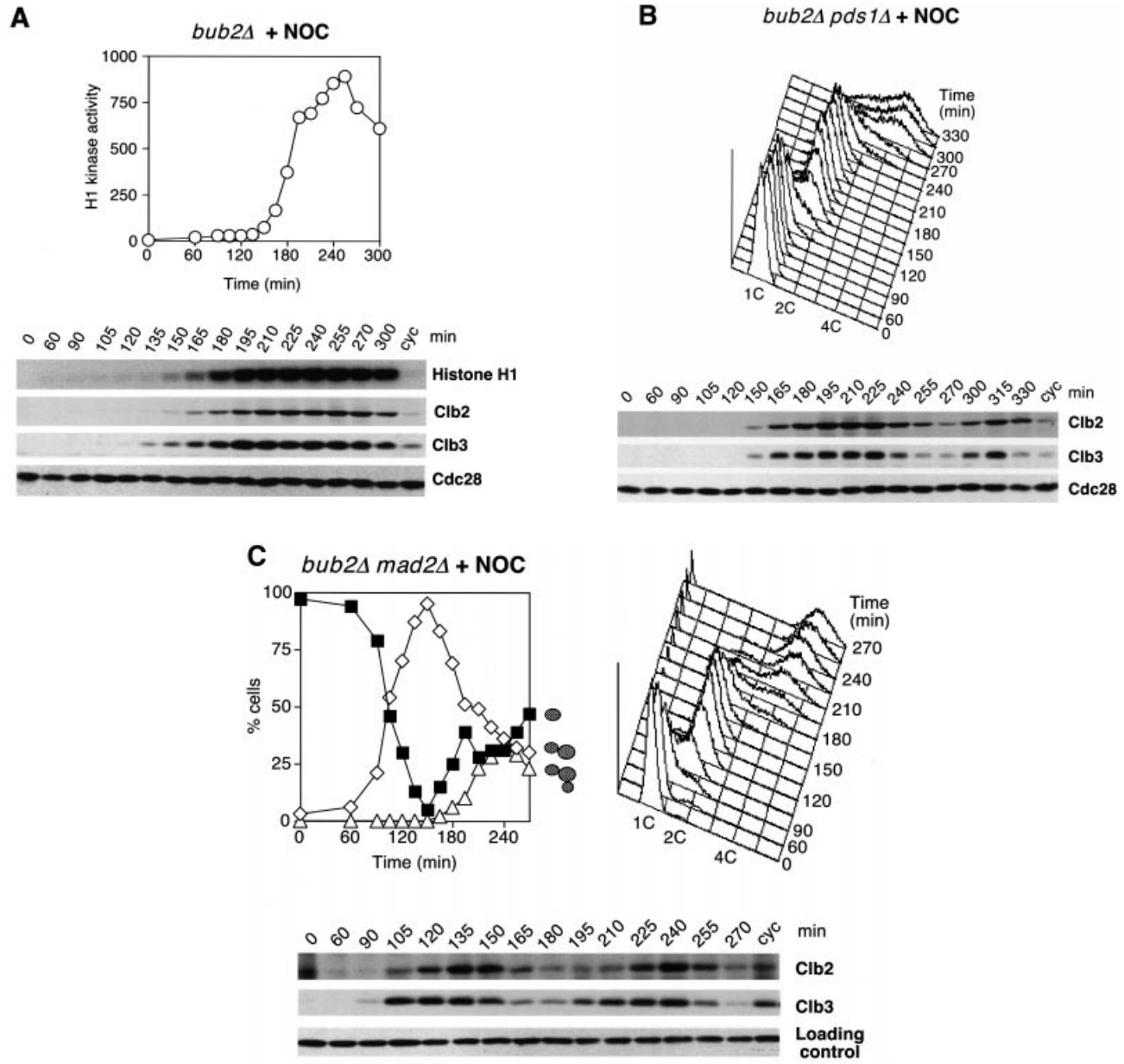


Fig. 6. Deletion of *BUB2* and *PDS1* or *BUB2* and *MAD2* causes cyclin degradation and DNA re-replication in cells treated with nocodazole. (**A**) Clb2 and Clb3 protein levels as well as Clb2–Cdk1 kinase activity are maintained at a high level upon incubation of elutriated *bub2Δ* cells in nocodazole. The graph shows quantification of the kinase activity on the substrate histone H1 (○). (B and C) Cellular DNA content and protein levels of Clb2 and Clb3 were analysed as G_1 cells of *bub2Δpds1Δ* (K7158) (**B**) and *bub2Δmad2Δ* (K7422) (**C**) double mutant strains progressed through the cell cycle in the presence of nocodazole. To measure cytokinesis of *bub2Δmad2Δ* double mutant cells, unbudded cells (■), cells with a single bud (◇), and cells with two buds ('re-budded' cells, △) were scored separately as shown in (C).

double mutant and *bub2mad2 cdc14-3* triple mutant cultures growing at 25°C. After 1 h, all three cultures were shifted from 25 to 36°C. Neither wild-type nor *mad2bub2 cdc14-3* triple mutant cells re-replicated their DNA, whereas the *mad2bub2* double mutants did (Figure 9A). This shows that loss of Mad2 and Bub2 does not bypass the need for Cdc14 during exit from mitosis. Cdc14 might therefore be Bub2's ultimate target.

Over-expression of TEM1 in mad2 mutant cells treated with nocodazole can bypass Bub2's block of DNA re-replication

Another protein needed in addition to Cdc14 for mitotic exit is a GTP-binding protein encoded by *TEM1*. It is therefore interesting that Bub2 shares sequence similarity with Cdc16 from *Schizosaccharomyces pombe*, a GTPase-activating protein (GAP) which regulates septum formation (Fankhauser *et al*., 1993). This raises the possibility that Bub2 inhibits Cdk1 inactivation by inducing hydrolysis of GTP bound to Tem1. To test this, we compared the re-replication kinetics in the presence of nocodazole of wild-type, *bub2mad2* double mutant and *mad2* single mutant cells expressing an additional *TEM1* gene from the *GAL* promoter. *TEM1* over-expression was induced by addition of galactose to cells previously grown in raffinose medium, immediately after addition of nocodazole. We observed that *TEM1* over-expression induced DNA re-replication in *mad2* mutant cells (Figure 9B).

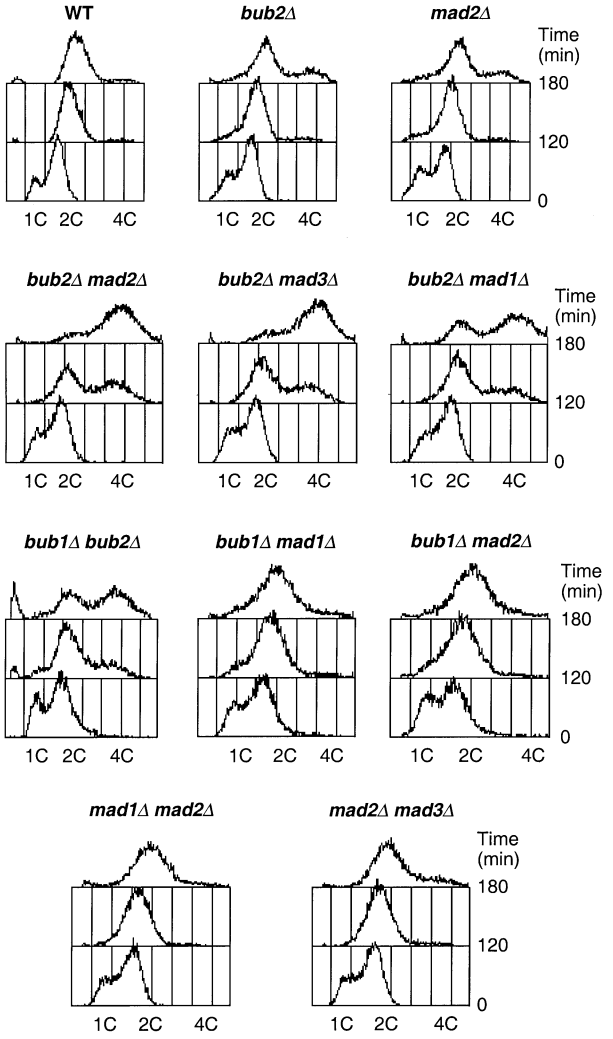


Fig. 7. Mad1, Mad2, Mad3 and Bub1 belong to a mitotic surveillance pathway distinct from the Bub2-dependent pathway. Strains were grown to exponential phase and cellular DNA was analysed at the indicated times after addition of nocodazole. The following strains were used: wild-type (K699), *bub2Δ* (K7145), *mad2Δ* (K7746), *bub2Δmad2Δ* (K7422), *bub2Δmad3Δ* (K7504), *bub2Δmad1Δ* (K7625), *bub1Δbub2Δ* (K7763), *bub1Δmad1Δ* (K7624), *bub1Δmad2Δ* (K7509), *mad1Δmad2Δ* (K7626), and *mad2Δmad3Δ* (K7501). *bub1Δmad1Δ, bub1Δmad2Δ, mad1Δmad2Δ* and *mad2Δmad3Δ* double mutants behaved similarly to wild-type, whereas *bub2Δmad3Δ* mutant cells re-replicated their DNA as efficiently as *bub2Δmad2Δ* mutant cells. Less efficient re-replication was observed in *bub2Δmad1Δ* and *bub1Δbub2Δ* mutant cells.

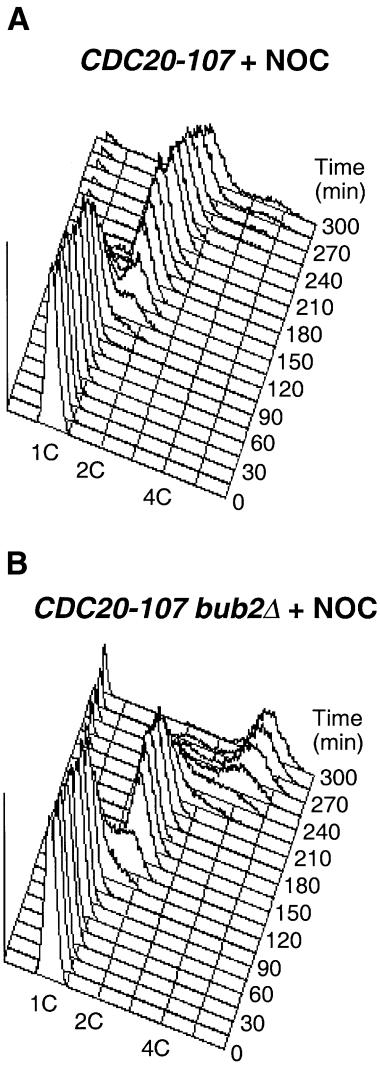


Fig. 8. *CDC20-107* mutants are defective only in the Mad2-dependent checkpoint pathway. Cellular DNA contents after incubation of elutriated *CDC20-107* (K8107) (**A**) and *CDC20-107 bub2Δ* (K8108) (**B**) cells in nocodazole. *CDC20-107* single mutant cells delay DNA re-replication for at least one generation time, while the *CDC20-107 bub2Δ* double mutants re-replicate with wild-type kinetics.

This is consistent with the hypothesis that Bub2 blocks Cdk1 inactivation by inhibiting Tem1 activity.

The budding yeast homologue of Byr4 is part of the Bub2 checkpoint pathway

byr4^{+} is an essential gene regulating karyokinesis and cytokinesis in *S.pombe* (Song *et al.*, 1996). Byr4 binds to Cdc16 and Spg1, the *S.pombe* homologues of Bub2 and Tem1, in yeast two-hybrid assays, and in coimmunoprecipitations *in vivo* and *in vitro* (Furge *et al.*, 1998; Jwa and Song, 1998). *In vitro*, Cdc16 and Byr4 form a two-component GAP for the Spg1 GTPase and they appear to negatively regulate septation in *S.pombe* by modulating the nucleotide state of Spg1 (Furge *et al.*, 1998). Looking for putative Byr4 homologues in the budding yeast by sequence homology search, we have found *YJR053W*, a previously uncharacterized open reading frame (ORF). Unlike the *S.pombe* gene, the *Saccharomyces cerevisiae* counterpart is not essential for cell viability. If *YJR053W* were a real homologue of *byr4*$^{+}$, one would expect it to have a role similar to *BUB2* in regulation of the spindle checkpoint. To test this, nocodazole was added to asynchronous cultures of *mad2bub2* double mutants, *byr4* single mutants and *byr4mad2* double mutants. After 3 h, *byr4* single mutants showed a moderate checkpoint defect as scored by DNA re-replication, while the *byr4mad2* double mutant cells exhibited the same strong checkpoint defect as *bub2mad2* double mutants. These results suggest that *YJR053W* is necessary for the Bub2-dependent cell cycle arrest. It therefore appears to have a role similar to *S.pombe byr4*$^{+}$, which prompted us to adopt the same name.

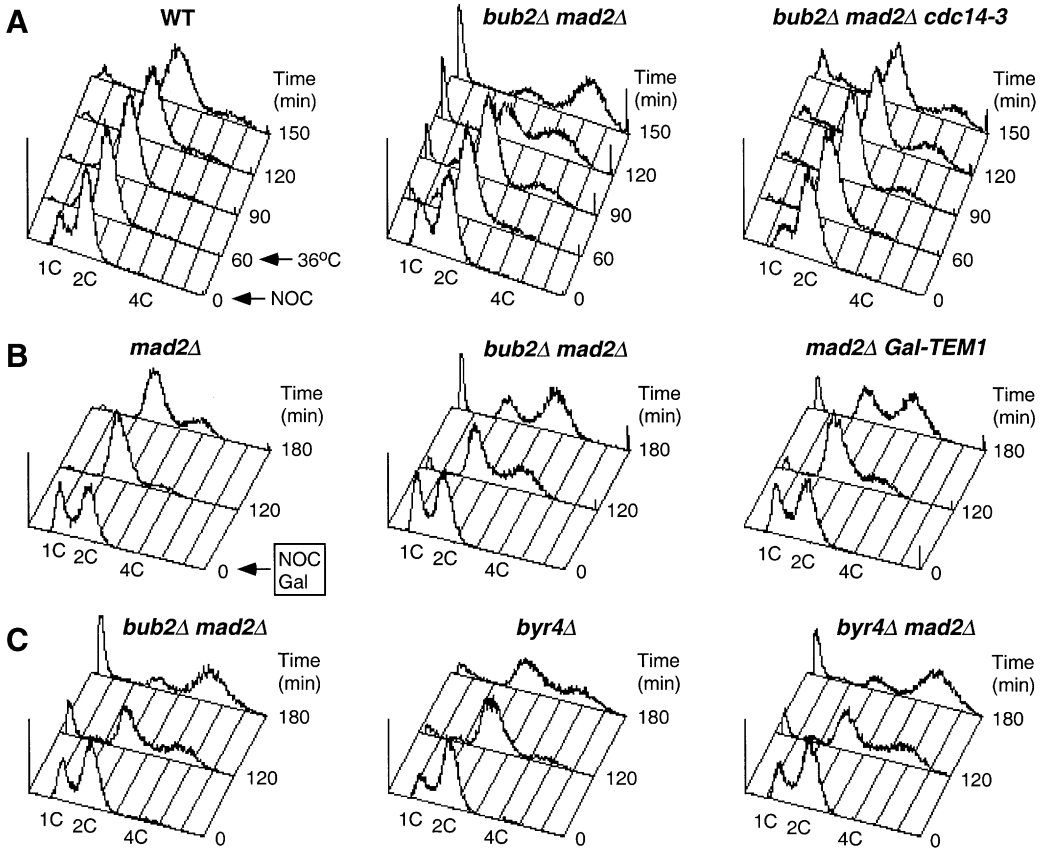


Fig. 9. Bub2 functionally interacts with Cdc14, Tem1 and the budding yeast homologue of Byr4. (**A**) DNA re-replication in *bub2Δmad2Δ* double mutant requires Cdc14 function. Exponentially growing cells of wild-type (K699), *bub2Δmad2Δ* (K7422) and *bub2Δmad2Δ cdc14-3* (K7744) were treated with nocodazole (15 μg/ml) at time point zero, and after 1 h the temperature was increased from 25 to 36°C in order to inactivate the temperature-sensitive *cdc14-3* allele. The cellular DNA content was measured by flow cytometry and it indicates that inactive Cdc14 can block DNA re-replication in cells lacking both Bub2 and Mad2. (**B**) *TEM1* over-expression allows DNA re-replication in *mad2* mutant cells treated with nocodazole. Exponentially growing *mad2Δ* (K7292) and *bub2Δmad2Δ* (K7422) cells and *mad2Δ* cells containing an additional *TEM1* gene expressed from the *GAL* promoter (K7765) were incubated in the presence of nocodazole (15 μg/ml) and the cellular DNA content was analysed at the time points indicated after nocodazole addition. *TEM1* over-expression, induced immediately after nocodazole treatment, allowed *mad2* mutant cells to re-replicate their DNA despite the Bub2 checkpoint activity. (**C**) The budding yeast homologue of the *S.pombe* gene *byr4*$^{+}$ is part of the Bub2 checkpoint pathway. Exponentially growing cells of *bub2Δmad2Δ* (K7764), *byr4Δ* (K8156) and *byr4Δmad2Δ* (K8157) were treated with nocodazole and the cellular DNA content was analysed at the time points indicated after nocodazole addition. Deletion of *BYR4* causes the same efficient DNA re-replication in *mad2* mutants as does deletion of *BUB2*.

Discussion

In the presence of lagging chromosomes or damaged spindles, most eukaryotic cells block separation of sister chromatids, inactivation of cyclin B–Cdk1 kinases, cytokinesis and chromosome re-duplication. They in effect arrest the cell cycle in a metaphase-like state. The existence of *mad* and *bub* mutants that fail to arrest the cell cycle under these circumstances suggested that specific 'surveillance mechanisms' or 'checkpoints' block the metaphase to anaphase transition when they detect chromosomes that have not formed bivalent attachments to the mitotic spindle. A resemblance between the mitotic arrest caused by lagging chromosomes or damaged spindles and that of mutants with defective APC subunits suggested that APC inhibition might be the means by which mitotic surveillance proteins block cell cycle progression.

Because of the belief that *mad* and *bub* mutants are defective in all aspects of the cell cycle arrest induced by spindle damage, it was supposed that these proteins might all be involved in a single pathway whose purpose was simply to inhibit the APC. However, it has recently become apparent that different APC substrates are degraded with the help of two different activators, WD40 proteins called Cdc20 and Cdh1. This raises the possibility that the two different forms of the APC, APC^{Cdc20} and APC^{Cdh1}, might be differently regulated.

Two distinct regulatory pathways regulate anaphase and mitotic exit

We show here that cell cycle arrest caused by disassembly of mitotic spindles in yeast can only be understood if we suppose that two distinct mitotic regulatory pathways exist (Figure 10). One pathway involving Mad1, Mad2, Mad3 and Bub1 inhibits APC^{Cdc20}, which blocks proteolysis of Pds1 and thereby prevents activation of the sister separating protein Esp1. This then is the mechanism by which cells block sister chromatid separation. In addition to inhibiting Esp1, Pds1 also blocks the onset of proteolysis mediated by the second APC activator, Cdh1. However, APC^{Cdh1} (and proteolysis of the B-type cyclin–Cdk1 inhibitor Sic1) is also regulated by a second, quite separate pathway whose function depends on Bub2. Due to this Bub2 pathway, cells lacking either Mad2 or Pds1 or even both proteins still arrest with high Clb2–Cdk1 kinase

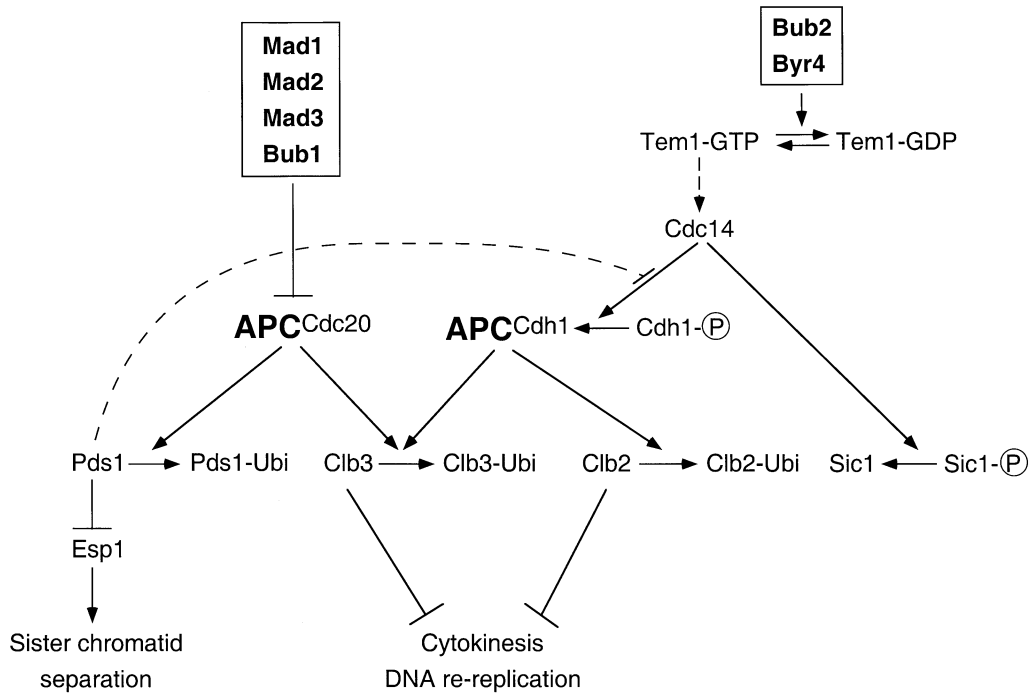


Fig. 10. A model proposing two surveillance pathways that respond to spindle damage. Mad2 and Bub2 function in different pathways and block distinct cell cycle events in response to nocodazole treatment. The 'Mad2' pathway, which involves Mad1, Mad2, Mad3 and Bub1, inhibits APC^{Cdc20} and thereby blocks Pds1 degradation and sister chromatid separation. This pathway also blocks degradation of Clb3 by APC^{Cdc20}. Pds1 has a second role, which is to block Clb2 degradation and Sic1 accumulation (it is not clear which is the actual target of Pds1 in this respect). The 'Bub2' pathway, which also includes Byr4, blocks cytokinesis and DNA re-replication by preventing inactivation of mitotic Cdk1 kinases, principally Clb2–Cdk1. We propose that Bub2 and Byr4 promote GTP hydrolysis by Tem1, which prevents accumulation of the GTP-bound Tem1 needed (by an unknown mechanism) for Cdc14 phosphatase activity. Cdc14 is possibly needed to de-phosphorylate and thereby 'activate' Cdh1, Swi5 and Sic1. It is proposed that Clb3 is degraded by both APC^{Cdc20} and APC^{Cdh1}. See text for further details.

activity. Only when cells lack both Mad2- and Bub2-dependent pathways, as in *bub2mad2* double mutants, do cells fully disregard spindle poisons such as nocodazole and charge through mitosis and enter the next cell cycle as if nothing were amiss.

We, and others before us (Hoyt *et al*., 1991; Minshull *et al*., 1996), have noticed that inactivation of either the Mad2 or the Bub2 pathway permits cells to exit mitosis after arrest for between one and two generation times. Both pathways are therefore required to maintain an indefinite mitotic arrest in yeast. This does not detract from the significance of our finding that Mad2 and Bub2 are largely concerned with blocking different aspects of the cell cycle. It is not unlikely that many, if not most, instances of cell cycle arrest encountered by cells are short in duration. Indeed, many if not most mammalian cells are only capable of arresting exit from mitosis for very limited periods of time.

Pds1's role in arresting the cell cycle

Until now the role of Pds1 has been one of the more confusing aspects of mitotic control in yeast. On the one hand, Pds1 clearly had a role in preventing the eventual separation of sister chromatids, in at least some cells treated with nocodazole (Yamamoto *et al*., 1996). On the other hand, Pds1 was not required for blocking inactivation of Cdk1 kinase (Yamamoto *et al*., 1996). These observations were difficult to reconcile with the model of a single regulatory pathway. However, they can be easily explained in the light of the scheme outlined in Figure 10.

Our finding that Scc1 dissociates from chromosomes with the same kinetics in *pds1* mutants in the presence of nocodazole as it does in the absence of this drug implies that Pds1 is crucial for delaying the loss of sister chromatid cohesion when spindles are damaged. Furthermore, we show for the first time that Mad2 is essential for delaying proteolysis of Pds1. These observations, along with the knowledge that Pds1 is an APC^{Cdc20} substrate (Visintin *et al*., 1997; Shirayama *et al*., 1998), that Mad proteins bind to APC^{Cdc20} (Fang *et al*., 1998; Hwang *et al*., 1998), that certain *CDC20* mutants cause a bypass of Mad2-dependent cell cycle arrest (Hwang *et al*., 1998; Schott and Hoyt, 1998) and that Pds1 binds the sister separating protein Esp1 (Ciosk *et al*., 1998), suggest that Mad proteins and Pds1 occupy very different places in a pathway concerned with blocking sister separation (see Figure 10). By binding to and inhibiting APC^{Cdc20}, Mad2 blocks the proteolysis of several proteins normally degraded by this form of the APC. This includes Clb3 (Figure 5D) and Clb5 (data not shown), in addition to Pds1. It is, however, the persistence of Pds1, and Pds1 alone, that is responsible for blocking sister separation, by tying up Esp1 in an inactive complex. Unlike Mad2, Pds1 is not required for blocking Clb3 proteolysis. This suggests that Pds1 is not required for the direct inhibition of APC^{Cdc20}. According to this scheme, the outlines of which were first proposed as a speculative model by Yamamoto *et al*. (1996), Pds1 clearly lies 'downstream' of the Mad proteins in a pathway that blocks sister separation in response to spindle damage.

Two pieces of evidence support the notion that Pds1 has a second role, which is to block APC^{Cdh1} (and

accumulation of Sic1). First, non-degradable versions of Pds1 block Clb2 proteolysis in addition to sister separation. Secondly, and more importantly, the block to Clb2 proteolysis that persists in *bub2* mutants treated with nocodazole depends on *PDS1*. This property of Pds1 provides a mechanism by which the Mad2 pathway inhibits APC^{Cdh1} as well as APC^{Cdc20}.

Mad2 is not required to delay Clb2–Cdk1 inactivation

The proposal that Bub2 acts in a separate pathway to Mad2 hinges on our observation that *mad2* mutants delay inactivation of Clb2–Cdk1 kinase and DNA re-replication for a period that corresponds to at least one generation time. Although our conclusions are novel, many of our observations on the behaviour of *mad2* mutants are in fact consistent with previously published data. Other studies have noted the failure of *mad2* mutants to maintain high levels of Cdk1 kinase activity (Li and Murray, 1991). Our finding along with that of Minshull *et al*. (1996), that *mad2* mutants cannot block the degradation of Clb3 (and Clb5), accounts for the drop in Cdk1 kinase activity. Furthermore, the Mad2 pathway clearly does help to maintain Clb2–Cdk1 kinase at high levels in cells treated with nocodazole, presumably through its indirect effect on Clb2 and Sic1 proteolysis via Pds1. This effect is possibly of biological significance, because a small fraction of *mad2* mutant cells do manage to exit from a mitotic state and re-replicate their genomes in the presence of nocodazole. The failure of other studies to notice the persistence of a mechanism which delays Clb2–Cdk1 inactivation in *mad2* mutants lies less with primary observations than in their interpretation. The use of induction synchrony, which generates cells much larger than normal, possibly reduces but does not eliminate the effect. Mad2 mutants do indeed re-replicate, albeit inefficiently, after long periods of incubation in nocodazole during which cells become very large. Cell size is a crucial factor in all cell cycle studies and many cell cycle blocks are overcome by the growth of cells. Earlier studies have ignored the persistence of significant levels of Clb2–Cdk1 kinase activity in *mad2* mutants incubated in the presence of nocodazole.

Bub2 blocks the cell cycle in mad2 mutants

Our ability to observe a robust cell cycle arrest in *mad2* mutants enabled us to investigate properly for the first time the role of Bub2 in blocking mitotic exit. The nocodazole-induced cell cycle arrest of *mad2* mutants is totally abolished by deletion of *BUB2* but not by that of *BUB1*, *MAD1* or *MAD3*. Our finding that cells only re-replicate efficiently in the presence of nocodazole if *BUB2* is deleted along with either *MAD1*, *MAD2*, *MAD3* or *BUB1* implies that Mad1, Mad2, Mad3 and Bub1 function in one pathway and Bub2 in another.

Bub2's mode of action

Several questions can be raised about the role of Bub2 in arresting the cell cycle. How does Bub2 mediate cell cycle arrest, what physiological process does it aim to prevent, and what sort of defects might it be responding to? Bub2 is not required for blocking Pds1 proteolysis due to APC^{Cdc20}, but it is essential, in the absence of Pds1, for blocking proteolysis of Clb2 and Cdc5 (data not shown). These proteins are degraded by APC^{Cdh1}, which implies that APC^{Cdh1} might be one of Bub2's targets. Cdh1's ability to bind to the APC, crucial for Clb2 ubiquitination, is inhibited by phosphorylation of Cdh1 by Cdk1 (Zachariae *et al*., 1998). Given the high levels of Clb2–Cdk1 kinase in *mad2* mutants arrested by nocodazole, we suspect that Cdh1 is hyper-phosphorylated and cannot therefore bind to the APC. If so, Bub2 must be necessary for preventing Cdh1 de-phosphorylation when spindles are damaged by nocodazole.

Bub2's role cannot, however, be confined to blocking Cdh1 de-phosphorylation, because *mad2* mutant cells also fail to accumulate the kinase inhibitor Sic1 as indicated by the maintenance of high Clb2–Cdk1 kinase activity. This could be achieved by an inhibition of *SIC1* transcription and persistent Sic1 proteolysis, both mediated by Clb2–Cdk1 (Moll *et al*., 1991; Knapp *et al*., 1996; Verma *et al*., 1997). The former could be due to a failure to de-phosphorylate the transcription factor Swi5 and the latter due to a failure to de-phosphorylate Sic1. Thus, Bub2 possibly prevents inactivation of Clb2–Cdk1 kinases by blocking de-phosphorylation of at least three proteins: Cdh1, Sic1 and Swi5.

One mechanism by which cells might normally escape from a self-sustaining high cyclin B–Cdk1 state would be to activate a phosphatase capable of de-phosphorylating Cdh1, Sic1 and Swi5. Cdc14 is a candidate for such a phosphatase. It is required for inactivating Clb2–Cdk1 and promoting Swi5 entry into nuclei during telophase and it has recently been shown to be capable of de-phosphorylating Swi5 and Sic1 *in vitro* (Visintin *et al*., 1998). We noticed, furthermore, that the re-replication of *bub2mad2* double mutants in the presence of nocodazole is abolished in *cdc14* mutants. We propose therefore that Bub2 acts by inhibiting activation of Cdc14, which would fully explain its maintenance of Clb2–Cdk1 kinase.

The sequence of Bub2 provides a clue as to how it might affect Cdc14. Bub2 shares sequence similarity with Cdc16, a GTPase-activating protein (GAP) that is required for regulating septum formation in *S.pombe* (Fankhauser *et al*., 1993). Bub2 is also homologous to two other GAPs from budding yeast, Gyp6 and Gyp7 (Neuwald, 1997). Cdc16 together with Byr4 form a two-component GAP for the Spg1 GTPase (Furge *et al*., 1998), which promotes septum formation (Schmidt *et al*., 1997). Spg1's *S.cerevisiae* homologue is a GTPase called Tem1, which is required along with Cdc14 for cytokinesis and exit from mitosis in budding yeast (Wan *et al*., 1992; Shirayama *et al*., 1994; Jaspersen *et al*., 1998). We therefore propose that activation of Cdc14 depends on a GTP-bound form of Tem1 and that when spindles are damaged, Bub2 activates Tem1 GTP hydrolysis, prevents accumulation of Tem1 in a GTP-bound form, and thereby inhibits Cdc14 activation. This hypothesis is consistent with our observation that Tem1 overproduction bypasses the Bub2-dependent block of DNA re-replication in *mad2* mutants treated with nocodazole. The existence of similar pathways which regulate cytokinesis and chromosome re-duplication in *S.pombe* and *S.cerevisiae* is further supported by identification of the budding yeast homologue of Byr4, which has a role similar to Bub2 in the cell cycle arrest induced by spindle damage. The main difference between the

S.pombe genes, *cdc16*$^+$ and *byr4*$^+$, and their counterparts in the budding yeast resides in the fact that the former are needed for regulating cytokinesis during undisturbed cell cycles and, therefore, they are essential genes (Fankhauser *et al*., 1993; Song *et al*., 1996), while the latter seem to be required only for the spindle checkpoint activity and they are not essential.

Regarding the physiological process regulated by Bub2 in response to spindle damage, we conclude that, unlike Mad2, Bub2 is not concerned with blocking sister chromatid separation, but rather the onset of cytokinesis and chromosome re-replication.

It is currently unclear what sort of defects the Bub2 pathway normally detects. Previous data suggesting that Bub2 is not required for mitotic arrest induced by low concentrations of nocodazole or by kinetochore mutants (Wang and Burke, 1995), indicate that the Bub2 pathway might not monitor spindle–kinetochore attachment but another defect caused by microtubule depolymerization such as the integrity of pole to pole spindles. Alternatively, the Bub2 pathway might detect spindle problems occurring after anaphase onset. A delay in cytokinesis could help chromatids not properly attached to the spindle to reach opposite poles of the cell. Bub2 could also monitor the integrity of cytoplasmic microtubules required for spindle orientation and nuclear positioning (Jacobs *et al*., 1988). Consistent with such a possibility, Bub2 was found to localize throughout the cell (M.Shirayama, personal communication).

Clb2, a late mitotic cyclin that regulates cytokinesis

Our observation that Clb3 is degraded in *mad2* mutants but not in *pds1* mutants in the presence of nocodazole can best be explained if the bulk of Clb3 were degraded by APC^{Cdc20}. Other data clearly demonstrate, however, that during G_1 Clb3 is degraded by APC^{Cdh1} (Zachariae *et al*., 1998). We propose that Clb3 may be a substrate for both APC^{Cdc20} and APC^{Cdh1} (see Figure 10). Thus, Clb3 degradation would be initiated by APC^{Cdc20} as cells enter anaphase and then taken over by APC^{Cdh1}. Clb2, on the other hand, can only be degraded by APC^{Cdh1}. In this regard, Clb3 behaviour resembles more closely the behaviour of B-type cyclins in animal cells than that of Clb2. This raises a question as to why yeast cells choose to delay Clb2 degradation until well after anaphase has been initiated. Its persistence might be important for preventing the premature onset of cytokinesis, which could be a problem peculiar to budding yeast cells that make many of the preparations for cell division very early in the cell cycle, when for example they generate septin rings at the bud neck soon after the initiation of DNA replication.

Materials and methods

Yeast strains and growth conditions

All strains were derivatives of W303 (also called K699: *MAT***a** *ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1*). Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) containing either 2% raffinose (YEPRaf) or 2% glucose (YEPD). To obtain synchronous cultures, cells were grown in YEPRaf medium at 25°C (or at 19°C in the case of *pds1Δ* strains) and small G_1 cells were isolated by centrifugal elutriation (Schwob and Nasmyth, 1993). G_1 cells were inoculated into YEPD medium at 25°C to a density of 7×10^6 cells/ml. Nocodazole was diluted into the medium to a final concentration of 15 μg/ml from a stock solution of 10 mg/ml in dimethylsulfoxide. *GAL-MPS1* strains were grown in YEPRaf medium at 21°C and the *GAL* promoter was pre-induced for 90 min by adding 3% galactose prior to elutriation. Small G_1 cells were released at 25°C into YEPRaf medium containing 3% galactose. For the experiment shown in Figure 5F, cells were arrested in G_1 at 25°C by incubation with α-factor (2.5 μg/ml) for 150 min and then released in medium containing nocodazole (15 μg/ml) and lacking α-factor. To induce *TEM1* expression from the *GAL* promoter, 2% galactose was added to a culture pregrown in YEPRaf.

Strain constructions

Strains containing Myc-tagged versions of *SCC1, PDS1* and *CLB2* have been described (Zachariae *et al*., 1996; Michaelis *et al*., 1997; Shirayama *et al*., 1998). The *BUB1* and *BUB2* ORFs were replaced by cassettes containing the *S.pombe his5*$^+$ gene which complements *his3* mutations in budding yeast. These cassettes were amplified by PCR with target gene-specific primers from pFA6a-HIS3MX6 (Wach *et al*., 1997) and transformed into yeast. The *MAD2, MAD3* and *BYR4* ORFs were replaced with cassettes containing the *Kluyveromyces lactis TRP1* gene. The *mad2* deletion strain used in Figure 5 was constructed using a *mad2::URA3* plasmid kindly provided by A.Murray. *MAD1* was disrupted by transformation with a *mad1::URA3* plasmid (Hardwick and Murray, 1995). The *GAL-MPS1* strain was described previously (Hardwick *et al*., 1996). The strain K8107 was constructed by transforming a *cdc20* deletion strain, kept alive by a plasmid bearing a *GAL* promoter-driven *CDC20* gene (Lim *et al*., 1998) with the *Hin*dIII fragment of a pCM4-derived plasmid carrying the *CDC20-107* allele (Hwang *et al*., 1998), followed by selection of colonies which lost the *GAL-CDC20* centromeric plasmid. *GAL-TEM1* construct (Shirayama *et al*., 1994) was integrated into the genome of the strain K7747 at the *ura3* locus. Genetic crosses and transformation of yeast strains were carried out according to standard protocols (Sherman *et al*., 1974).

Immunoblot analysis and histone H1 kinase assay

Protein immunoblot analysis was performed as described (Surana *et al*., 1993). After transfer to Immobilon P membranes (Millipore), proteins were detected using an enhanced chemiluminescence detection system (ECL, Amersham). Clb2 and Cdc28 were detected using polyclonal rabbit antisera diluted 1:3000. Rabbit affinity-purified Clb3 antibodies (kindly provided by D.Kellogg) were used at a dilution of 1:1500. In experiments in which both Western blotting and H1 kinase assays were performed, the extracts prepared for the kinase assay were also used for Western blotting. Histone H1 kinase assays were performed as described previously (Surana *et al*., 1993). Phosphorylated histone H1 was detected by autoradiography and quantified with a Molecular Dynamics PhosphoImager using the ImageQuant software.

Other techniques

The DNA content of cells stained with propidium iodide was measured on a Becton Dickinson FACScan flow cytometer as described (Epstein and Cross, 1992). Chromosomes were visualized in cells expressing a *tet* repressor–GFP fusion protein which binds to an array of *tet* operators integrated at the *ura3* locus, 35 kb from the centromere of chromosome V (Michaelis *et al*., 1997). Chromosome spreading and visualization of yeast chromosomes using the *tet*R–GFP/*tet* operators system were performed as described previously (Michaelis *et al*., 1997). Cells were prepared for indirect immunofluorescence according to Nasmyth *et al.* (1990). Myc-tagged proteins were detected using 9E10 hybridoma supernatant diluted 1:5 and a CY3-conjugated secondary antibody. Spindles were detected using a rabbit anti-yeast tubulin antibody and a FITC-conjugated secondary antibody. DNA was stained with DAPI.

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