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A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast

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Exit from mitosis in all eukaryotes requires inactivation of the mitotic kinase. This occurs principally by ubiquitin-mediated proteolysis of the cyclin subunit controlled by the anaphase-promoting complex (APC). However, an abnormal spindle and/or unattached kinetochores activates a conserved spindle checkpoint that blocks APC function. This leads to high mitotic kinase activity and prevents mitotic exit. *DBF2* belongs to a group of budding yeast cell cycle genes that when mutated prevent cyclin degradation and block exit from mitosis. *DBF2* encodes a protein kinase which is cell cycle regulated, peaking in metaphase–anaphase B/telophase, but its function remains unknown. Here, we show the Dbf2p kinase activity to be a target of the spindle checkpoint. It is controlled specifically by Bub2p, one of the checkpoint components that is conserved in fission yeast and higher eukaryotic cells. Significantly, in budding yeast, Bub2p shows few genetic or biochemical interactions with other members of the spindle checkpoint. Our data now point to the protein kinase Mps1p triggering a new parallel branch of the spindle checkpoint in which Bub2p blocks Dbf2p function.

Keywords: Bub2p/cell cycle/Dbf2p kinase/*Saccharomyces cerevisiae*/spindle checkpoint

Introduction

In the budding yeast *Saccharomyces cerevisiae*, Cdc28p is the major cyclin-dependent kinase. It associates with various Clb cyclins, principally Clb2p, to form the kinase that controls mitosis. Inactivation of this mitotic kinase is essential for exit from mitosis (reviewed in Nasmyth, 1996); indeed this is true in all eukaryotes. This inactivation occurs principally by ubiquitin-mediated proteolysis of the cyclin subunit. E3 ubiquitin ligases (together with substrate-specific adaptor proteins) are crucial in specifying the selection of proteins for this proteolysis. Recent studies in human, *Xenopus* and yeast systems have converged to demonstrate a striking conservation in structure of the E3 activity that acts in late mitosis (reviewed in Page and Hieter, 1997). This E3 is a complex of some 12 proteins and is known as the cyclosome or anaphase-promoting complex (APC) (Zachariae *et al.*, 1996, 1998).

As its name suggests, an active APC is required for cells to initiate anaphase, and strains defective in APC function arrest in metaphase (Kramer *et al.*, 1998; Zachariae *et al.*, 1998). Clb2p degradation normally is initiated at anaphase onset, but its degradation is not required for the metaphase to anaphase transition (Ghiara *et al.*, 1991; Holloway *et al.*, 1993) and some Clb2p persists until telophase (Surana *et al.*, 1993). Clearly APC must have other critical targets. One of the early events required for the metaphase to anaphase transition is sister chromatid separation. Several proteins whose proteolysis is required for sister chromatid separation have been identified, e.g. Cut2p in fission yeast (Funabiki *et al.*, 1996) and Pds1p in budding yeast (Cohen-Fix *et al.*, 1996; Yamamoto *et al.*, 1996a,b). Yeast strains lacking *PDS1* are viable but, without Pds1p, APC-deficient mutants no longer show a predominant metaphase arrest. Instead, a large proportion of cells now arrest in telophase with divided chromatin (Yamamoto *et al.*, 1996a; Kramer *et al.*, 1998). APC therefore has a critical role in sister chromatid separation as well as in mitotic kinase inactivation.

The mechanisms controlling APC function are complex and involve phosphorylation of APC subunits as well as direct binding of regulatory factors to the core APC. The spindle checkpoint also regulates APC function (Rudner and Murray, 1996; Hardwick, 1998). This is activated by an abnormal spindle and/or unattached kinetochores, and results in a cell cycle arrest at metaphase. The checkpoint is a pathway consisting principally of the Mad and Bub proteins (see Figure 7A) (Hoyt *et al.*, 1991; Li and Murray, 1991). Bub1p is a protein kinase that is complexed with Bub3p, a possible regulator of Bub1, and Mad1p and Mad2p also form a complex (Roberts *et al.*, 1994; Chen *et al.*, 1998; Hwang *et al.*, 1998; Taylor *et al.*, 1998). Sequence homology between Mad3p and Bub1p suggests that Mad3p may intereact with the Bub1p–Bub3p complex. Bub2p is not known to associate with any of these proteins and displays certain functional differences from them (see Discussion). A second protein kinase, Mps1p, appears to be located near the top of the pathway. Overexpression of Mps1p in the absence of spindle damage is sufficient for cell cycle arrest, and this is dependent upon the *MAD* and *BUB* genes (Hardwick *et al.*, 1996). Importantly, these Mad and Bub proteins are conserved from yeast to metazoans so that the spindle checkpoint is one of the core cell cycle pathways (Chen *et al.*, 1998; Fang *et al.*, 1998; Taylor *et al.*, 1998). One immediate target of the spindle checkpoint is Cdc20p, one of the substrate-specific adaptors for APC (Schwab *et al.*, 1997; Lorca *et al.*,

1998). Cdc20p primarily controls Pds1p degradation (Visintin *et al.*, 1997; Hwang *et al.*, 1998; Lim *et al.*, 1998). Hence, Pds1p remains stable in cells arrested by means of the spindle checkpoint, leading to the observed metaphase arrest.

Apart from the spindle checkpoint, in budding yeast many other genes control mitotic progression and in particular the end of mitosis. Those controlling mitotic exit include genes encoding many signalling proteins such as protein kinases, a protein phosphatase and GTP-binding proteins (reviewed in Deshaies, 1997). Moreover, these genes show many genetic interactions (for example, see Kitada *et al.*, 1993; Toyn and Johnston, 1993; Jaspersen *et al.*, 1998), suggesting that they form a regulatory network (the mitotic exit network or MEN). The function of this network is not clear, but an obvious possibility is that it controls APC activity. Recent work provides indirect evidence for this. Mutations in many of the genes constituting the MEN are defective in ubiquitination of cyclin *in vitro* (Charles *et al.*, 1998; Jaspersen *et al.*, 1998). The best evidence for APC control by one of the MEN genes concerns Cdc5p. This is a polo-like protein kinase (Kitada *et al.*, 1993) that acts late in mitosis and is required specifically for degradation of Clb2p by the APC but not for degradation of Pds1p (Charles *et al.*, 1998; Kotani *et al.*, 1998; Shirayama *et al.*, 1998).

Other protein kinases that are part of the MEN include Dbf2p and Dbf20p. Temperature-sensitive mutants of *DBF2* arrest in telophase with an extended spindle and divided chromatin (Johnston *et al.*, 1990; Toyn and Johnston, 1994). The gene is expressed under cell cycle control during mitosis and the kinase activity is also cell cycle regulated. The peak of kinase activity occurs at about the time when division of chromatin is initiated (Toyn and Johnston, 1994), indicating a late mitotic function. Execution point analysis and the cell cycle arrest point of the mutant are also consistent with this. *DBF2* shows genetic interactions with *CDC5* and also with *CDC15*, another MEN protein kinase, in that multiple copies of the latter genes can suppress *dbf2^{ts}* mutants (Kitada *et al.*, 1993).

Rather surprisingly, strains deleted for *DBF2* are viable. This is due to the existence of *DBF20* which encodes a protein highly homologous to Dbf2p (Toyn *et al.*, 1991). Deletion of both *DBF2* and *DBF20* is lethal so that between them the two genes carry out an essential late mitotic function. The reason why Dbf20p does not carry out this function in a *dbf2^{ts}* strain may be that Dbf2p in some way sequesters Spo12p. This protein appears to be rate limiting for Dbf2p function and is required by Dbf20p if it is to replace Dbf2p functionally (Parkes and Johnston, 1992; Toyn and Johnston, 1993). The substrates of Dbf2p are not known, and its precise cell cycle function is also not clear.

To help to understand Dbf2p function, we initially examined the timing of its activation more precisely. It is activated after Cdc5p and downstream of the spindle checkpoint. Indeed, we found Dbf2p to be negatively regulated by the spindle checkpoint, specifically by Bub2p. Bub2p and Dbf2p are part of a novel branch of the checkpoint that is also controlled by Mps1p.

Table I. Genotypes of strains

Strain name	Genotype	Source
W303-1A	MAT**a** *ura3-1,trp1-1, ade2-1,leu2-3,112, his3-11,15*	
YDF13	*W303-1A TRP1::DBF2-3MYC,URA3::CDC5-3HA*	this study
CG378	*Mat**a** ura3 leu2 trp1 ade5*	
CG379	*Matα ura3 leu2 trp1 his7*	
KTM208	CG378 *apc2:HIS7* p*apc2-8*	Kramer *et al.* (1998)
YDF20	KTM208 *URA3::DBF2-6MYC*	this study
YDF21	KTM208 *URA3::DBF2-6MYC mad2::LEU2*	this study
YDF22	KTM208 *URA3::DBF2-6MYC bub2::LEU2*	this study
YDF24	KTM208 *URA3::DBF2-6MYC bub3::LEU2*	this study
KTM308	KTM208 *pds1::URA3*	Kramer *et al.* (1998)
YDF31	YDF20 *BUB2::Kan MX4*	this study
YDF32	KTM308 *LEU2::DBF2-6MYC*	this study
YDF37	CG378 *mad2::LEU2*	this study
YDF38	CG379 *bub2::TRP1*	this study

Results

Dbf2p kinase is activated during metaphase–anaphase after the Cdc5p kinase activity

We previously have shown Dbf2p kinase activity to be expressed under cell cycle control during mitosis (Toyn and Johnston, 1994). To gain further insight into Dbf2p function, we determined more exactly where it is activated with respect to other late mitotic events. Initially, we compared Dbf2p activation with that of the Cdc5p kinase and the mitotic kinase. Cdc5p acts late in mitosis and controls Clb2p degradation through regulating APC function (Shirayama *et al.*, 1998). We constructed a *Mat**a*** strain, YDF13 (Table I), containing epitope-tagged Cdc5p and Dbf2p expressed from their own promoters. Both were tagged at their C-terminus, Cdc5p with triple hemagglutinin (HA) and Dbf2p with triple Myc, and both were fully functional (data not shown). This epitope-tagging strategy ensured that we measured only the Dbf2p-dependent kinase without interference from the kinase activity of its homologue Dbf20p (Toyn and Johnston, 1994). In a culture synchronized by α-factor, the Dbf2p protein was stable across the cell cycle whilst Cdc5 fluctuated in level (Figure 1; Shirayama *et al.*, 1998). However, activation of the Cdc5p kinase clearly precedes that of the Dbf2p kinase by some 15–20 min (Figure 1). Mitotic kinase activity was measured in the same culture, and Dbf2p kinase reached a peak as the mitotic kinase (specifically Clb2 kinase) started its sharp decline. This coincided with the degradation and loss of the Clb2p protein itself (Figure 1).

The timing and duration of the Dbf2p kinase suggested that it would be active from metaphase through to anaphase/telophase. Note that we use the terms metaphase and anaphase to refer to the state of the cell cycle machinery rather than to the cytological state of the cells, which has not been determined. Supporting this conclusion concerning activation of Dbf2p, in cell cycle arrest experiments the Dbf2p kinase activity is low in G_1 (α-factor arrest) and also in S-phase (hydroxyurea arrest) (data not shown). However, it is high in metaphase-arrested cells.

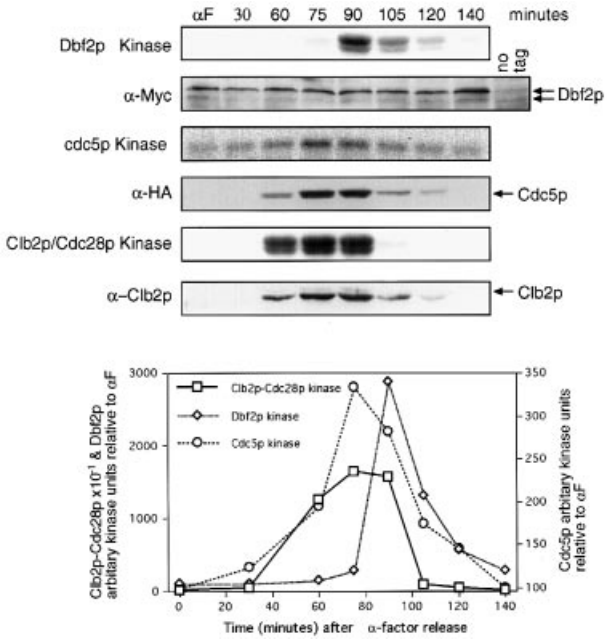

Fig. 1. Dbf2p kinase is activated after Cdc5p kinase. Strain YDF13 carrying integrated *DBF2-3MYC* and *CDC5-3HA* was arrested with α-factor for 3 h and released at 30°C. Samples for protein extracts were collected at the indicated times after release. To ensure that we observed the time course of Dbf2p kinase activation relative to the initiation of cyclin degradation, α-factor was added 75 min after the release. Protein levels and immunoprecipitated kinase activity were measured in parallel in the same extract: Dbf2p kinase and Dbf2p (upper panels); Cdc5p kinase and Cdc5p (middle panels); and Clb2p–Cdc28p kinase and Clb2p (lower panels). The graph shows a phosphoimager quantitation of kinase activity expressed relative to the respective activity observed in α-factor-arrested cells.

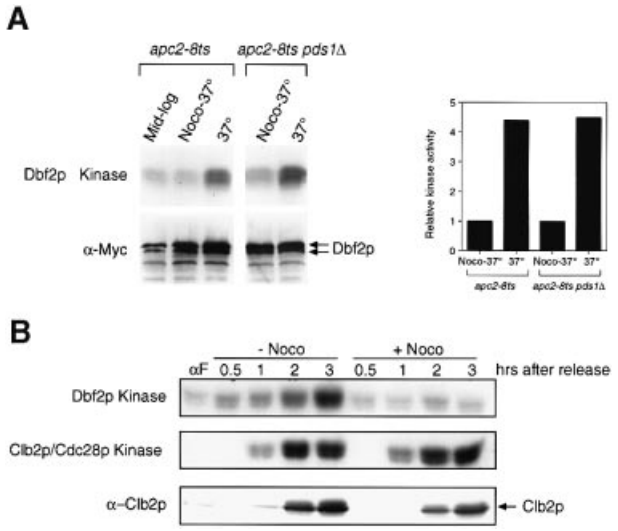

Fig. 2. Activating the spindle checkpoint prevents Dbf2p kinase activation. (**A**) Isogenic strains YDF20 (*apc2-8^{ts}*) and YDF32 (*apc2-8^{ts} pds1Δ*) (Table I) both carrying an integrated *DBF2-6MYC* were treated with nocodazole at 37°C (Noco-37°C) or simply incubated at 37°C for 3 h (37°C). Immunoprecipitated Dbf2p (lower panel) and Dbf2p kinase (upper panel) were analysed by anti-Myc (α-MYC) Western blot and autoradiography, respectively. The graph shows a phosphoimager quantitation of Dbf2p kinase activity relative to the nocodazole-arrested cells. (**B**) Strain YDF20 (*apc2-8 DBF2-6 MYC*) was arrested for 3 h with α-factor and released at 37°C in the presence (+Noco) or absence (–Noco) of nocodazole. Samples for protein extracts and FACS analysis (not shown) were taken at the indicated times after release. Extracts were prepared and immunoprecipitated or Western blotted with antibodies against Myc for Dbf2p kinase or against Clb2p (α-Clb2p). Dbf2p and Clb2p immunoprecipitates were subjected to protein kinase assay. αF, extracts from α-factor-arrested cells.

To demonstrate this, we assayed the kinase during incubation of an *apc2^{ts}* mutant at 37°C. We used an *apc2-8* temperature-sensitive mutant containing a 6Myc epitope-tagged Dbf2p (YDF20). Such mutants arrest in metaphase with stable Clb cyclin (Kramer *et al.*, 1998). Following a 3 h incubation of *apc2-8* mutant cells at the restrictive temperature, we reproducibly detected high levels of Dbf2p kinase activity in immunoprecipitates (Figure 2A). Deletion of *PDS1*, a metaphase target of the APC (see Introduction), allows arrested *apc* mutants, including *apc2-8*, to traverse metaphase (Yamamoto *et al.*, 1996a; Kramer *et al.*, 1998). Separation of chromatin occurs, resulting in final arrest of most cells in anaphase B or telophase. To determine whether Dbf2p kinase remained high in anaphase/telophase, we measured the activity at 37°C in our *apc2-8* strain containing a deletion of *PDS1* (Figure 2A). There was no change in the Dbf2p kinase activity compared with the *apc2-8* mutation alone.

In summary, Dbf2p activity is high during the metaphase–anaphase/telophase interval, the peak coinciding with initiation of Clb2p degradation. This is consistent with our previous data showing Dbf2p kinase reaching a peak at around the time of chromatin division (Toyn and Johnston, 1994). Taken together with the activation of Dbf2p after Cdc5p, this points to a late mitotic function for the Dbf2p kinase at about the time when inactivation of the mitotic kinase begins.

Dbf2p acts downstream of the spindle checkpoint

The maximum Dbf2p kinase activity coincides with the time of Clb2p kinase inactivation (Figure 1). This would be consistent with Dbf2p acting to control APC function, and recent data support this notion (Jaspersen *et al.*, 1998). Another late pathway regulating APC function is the spindle checkpoint. We therefore explored whether this checkpoint-mediated control of APC activity could involve Dbf2p. We initially did this by carrying out a reciprocal shift experiment between the nocodazole- and *dbf2* temperature-sensitive arrest points. Nocodazole disrupts the spindle and so activates the spindle checkpoint. This experiment therefore assesses the timing of Dbf2p function with respect to this checkpoint.

A *dbf2^{ts}* strain was treated with nocodazole for one generation at 25°C. The drug was then removed and incubation of the cells was continued at either 25 or 37°C. The culture at 37°C was unable to divide further (Figure 3A). Hence, nocodazole must act before the *dbf2^{ts}* arrest point. If it were after this arrest point, in the absence of nocodazole the cells would have been able to complete the cell cycle and divide once at 37°C. In the reciprocal experiment, a culture initially arrested at 37°C was transferred to 25°C in the presence or absence of nocodazole. The cells incubated in the presence of the drug were still able to divide once (Figure 3B). Thus, Dbf2p must act after the nocodazole arrest point. If Dbf2p function preceded the nocodazole arrest point, upon resuspension

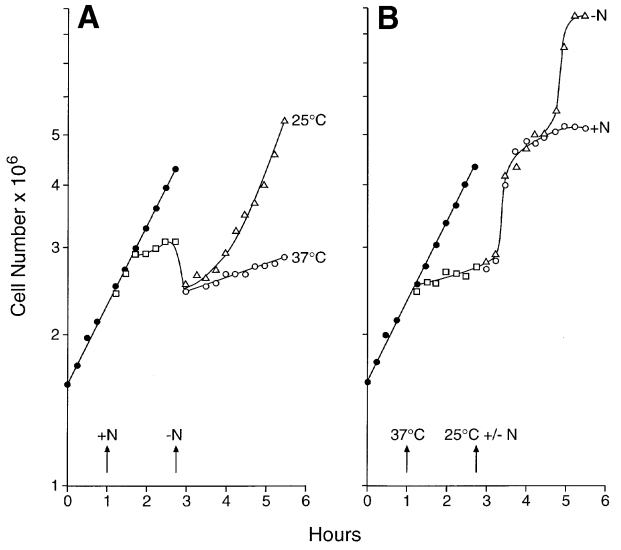

Fig. 3. Dbf2p acts downstream of the spindle checkpoint. (**A**) A mid-log culture of a *dbf2-2*^{ts} strain, J2 (Toyn and Johnston, 1993), growing at 25°C in YEPD was arrested by addition of 15 μg/ml nocodazole. After one generation, the cells were harvested, washed with YEPD and resuspended in YEPD at either 25 or 37°C. ●, control culture at 25°C; □, culture with addition of nocodazole; △, removal of nocodazole and transfer to 25°C; ○, removal of nocodazole and transfer to 37°C. (**B**) The mid-log cells were transferred to 37°C for one generation and then returned to 25°C in either the presence or absence of nocodazole. ●, control culture at 25°C; □, culture transferred to 37°C; △, culture returned to 25°C without nocodazole; ○, culture transferred to 25°C with nocodazole. +N, –N and +/–N, addition or removal of nocodazole. Cells numbers were determined by use of a particle counter (Coulter Electronics, Dunstable, UK).

in the drug, no further division could have occurred. Hence, Dbf2p function is downstream of the nocodazole arrest point.

The above division of the *dbf2*^{ts} arrested cells upon return to 25°C in the presence of nocodazole is a rather surprising observation. The *dbf2*^{ts} arrest results in cells with intact and elongated spindles (Toyn and Johnston, 1994). Presumably, the nocodazole disrupts these spindles, yet the cells still divide (Figure 3B). The simplest explanation is that the spindle checkpoint can no longer be activated once cells reach the *dbf2*^{ts} arrest point. This reciprocal shift analysis shows that Dbf2p clearly functions after the nocodazole arrest point and hence downstream of the spindle checkpoint. Dbf2p is therefore unlikely to be part of the spindle checkpoint itself, which would have resulted in inter-dependence of the *dbf2*^{ts} and nocodazole arrests, but potentially could itself be a target of the checkpoint.

Dbf2p kinase activation is impaired in spindle checkpoint-arrested cells

In view of Dbf2p acting downstream of the spindle checkpoint, we considered the possibility that it could be controlled by the checkpoint. To explore this, we employed the *apc2-8* temperature-sensitive arrest described above (Figure 2A). The extent of the Dbf2p activation at 37°C in the *apc2-8* and *apc2-8 pds1Δ* strains presumably reflects the high kinase activity seen in the cell cycle which occurs in the metaphase–anaphase/telophase interval (Figure 1). In fact the level in the *apc2-8* arrest is somewhat higher than the peak level in synchronized cells (not shown). This is to be expected given that the entire population in the *apc2-8* arrest is expressing the kinase, whereas synchrony is far from perfect. The levels of Dbf2p kinase in *apc2-8* arrested cells are also similar to those in *cdc14* cells at 37°C (not shown), a mutant that arrests in telophase. Thus, the Dbf2p kinase levels in *apc2-8* arrested cells reflect *in vivo* levels during mitosis, and this arrest can be used to investigate control of the kinase.

Initially, *apc2-8* mid-log cells were transferred to 37°C in the presence of nocodazole which, of course, activates the spindle checkpoint. In the presence of nocodazole, the Dbf2p kinase activity remained low (Figure 2A). This inhibitory effect of nocodazole was also apparent in the *apc-2 pds1Δ* cells. The spindle checkpoint targets Pds1p (see Introduction) which leads to a metaphase arrest. Significantly, this is the same point at which the *apc2-8* strain arrests at 37°C (Kramer *et al.*, 1998). However, in sharp contrast to cells treated with nocodazole, the Dbf2p kinase activity is high in *apc2-8* cells at 37°C (Figure 2A). This inhibitory effect of nocodazole is also apparent in the *apc2-8 pds1Δ* cells. Hence, nocodazole in some way prevents activation of the Dbf2p kinase activity.

To confirm the above data and rule out the possibility that the nocodazole arrest could have occurred after the peak in Dbf2p kinase activity, we examined a synchronized culture. The *apc2-8* strain grown at 25°C was released from α-factor arrest and we followed the Dbf2p kinase activity at 37°C in the presence or absence of nocodazole (Figure 2B). In both cases, the cells arrested with a 2C DNA content (data not shown) and Clb2p accumulated with a concomitant increase in Clb2p–Cdc28p kinase activity. Dbf2p kinase activity also accumulated in the absence of nocodazole. However, in the presence of the drug, only a low background level of the Dbf2p kinase is detectable. Western blot analysis showed no change in Dbf2p levels, with or without nocodazole (data not shown, but see Figure 2A). This inhibition of Dbf2p activation by treatment of cells with nocodazole depended upon activation of the checkpoint. When we allowed the activation of Dbf2p by simply incubating *apc2-8* cells at 37°C and then added nocodazole to the culture, there was no effect on the Dbf2p kinase activity (data not shown). In addition, no inhibitors of Dbf2p were detected in extracts from nocodazole-treated cells (data not shown). Thus, the spindle checkpoint cannot inhibit Dbf2p once it is activated, rather the checkpoint prevents activation of the Dbf2p kinase.

Dbf2p kinase activation is negatively regulated by Bub2p

If the spindle checkpoint does indeed control Dbf2p kinase activation, mutation of one or other of the *MAD* or *BUB* genes should alleviate the nocodazole-induced block in Dbf2p kinase activation. The Mad1p and Mad2p proteins form a complex, as do Bub1p and Bub3p (see Figure 7A), whereas Bub2p is not known to associate with either complex. Accordingly, we examined the effect of deleting *MAD2*, *BUB3* or *BUB2* on Dbf2p kinase activation in the presence of nocodazole. Once again this was done in an *apc2-8* genetic background to ensure a uniform arrest of all cells. Deletion of *MAD2* and *BUB3* was without effect on Dbf2p kinase activation (Figure 4), i.e. even in the absence of these genes and consequent ablation of the

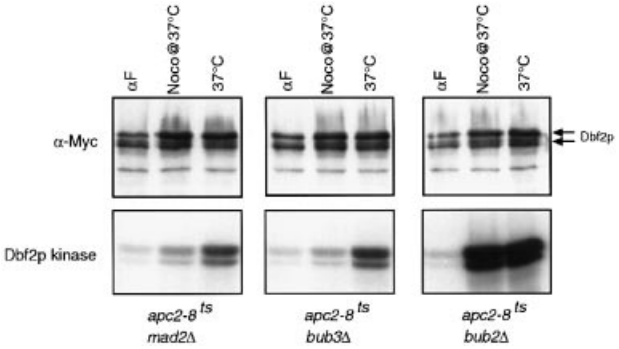

Fig. 4. Bub2p negatively controls Dbf2p activation. Isogenic strains carrying integrated *DBF2*-6MYC, YDF20 (*apc2-8*^{ts}), YDF21 (*apc2-8*^{ts} *mad2Δ*), YDF22 (*apc2-8*^{ts} *bub2Δ*) and YDF24 (*apc2-8*^{ts} *bub3Δ*) were grown to mid-log phase at 25°C and arrested with α-factor (αF) or with nocodazole at 37°C (Noco@37°C) or at 37°C (37°C) for 3 h. Extracts were prepared and subjected to immunoprecipitation and kinase assay (lower panel). The upper part of the kinase assay gel was Western blotted with anti-Myc antibody to control for even pull-down of Dbf2p (upper panel).

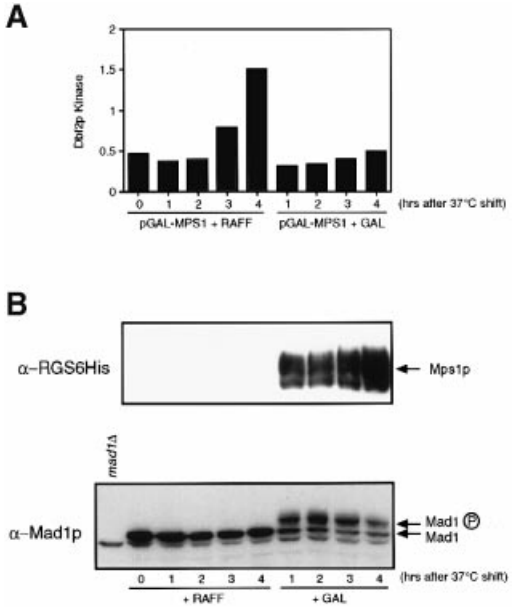

Fig. 5. Mps1p overexpression prevents Dbf2p kinase activation. Strain YDF20 (*apc2-8 DBF2-6MYC)* containing a plasmid expressing a 6His-T7-tagged Mps1p under the control of the *GAL10* promoter was grown to mid-log phase at 25°C in YEP 2% raffinose (repressed state); the culture was then split and to one half was added galactose (induced state) to 2% final concentration. Both cultures were incubated for a further 45 min at 25°C then shifted to 37°C. Samples were collected every hour post-shift for protein extract and FACS analysis (not shown). Dbf2p kinase activity was measured as in Figure 2. (**A**) Kinase activity was plotted relative to the Dbf2p protein levels in the pull-down (see Figure 4). (**B**) The corresponding extracts from the non-induced and induced cultures as well as from a *mad1Δ* (*mad1Δ*) strain, were Western blotted with anti-RGS-6His to detect overexpressed Mps1p (α-RGS-6His, upper panel) and with anti-Mad1p to detect the mobility shift of phosphorylated Mad1p (arrows, α-Mad1p, lower panel).

checkpoint, nocodazole still prevented Dbf2p kinase activation. In sharp contrast, deletion of *BUB2* led to deregulation of Dbf2p kinase activity and hyperactivation in the presence of nocodazole. There was an ~20-fold increase compared with the other strains treated with nocodazole (Figure 4). Even in the absence of nocodazole, hyperactivation of Dbf2p kinase occurred in the *bub2Δ* strain. However, this hyperactivation was not observed in exponentially growing *bub2Δ* cells at 25°C (data not shown). A Bub2p-dependent pathway that seems not to involve the Bub1p–Bub3p or Mad protein complexes negatively controls Dbf2p kinase activation.

The Mps1p kinase controls Dbf2p activation

Since deletion of *BUB2* alone leads to deregulation of Dbf2p kinase activity, the spindle checkpoint is unlikely to be a simple linear pathway that includes Bub2p. Bub2p may lie in a pathway independent of that containing the remaining *MAD* and *BUB* genes or it might form a separate branch of that pathway. To explore this, we used overexpression of the Mps1p protein kinase. This leads to a *MAD*- and *BUB*-dependent activation of the spindle checkpoint without spindle damage (Hardwick *et al.*, 1996), placing Mps1p near the top of the pathway (see Figure 7A). We reasoned that if Bub2p–Dbf2p were a branch of the pathway, rather than functioning independently, hyperactivation of Mps1p should prevent Dbf2p kinase activation. This is indeed what we observed (Figure 5). Mps1p overexpression led to the expected hyperphosphorylation of Mad1p (Hardwick and Murray, 1995) and no activation of the Dbf2p kinase occurred. When *BUB2* was deleted from this strain, the activation of Dbf2p kinase was once again observed (data not shown). This is the expected result since the metaphase arrest induced by Mps1p overexpression is known to be Bub2p-dependent (Hardwick *et al.*, 1996). The above data strongly suggest that Mps1p controls Dbf2p kinase activation through Bub2p.

BUB2 and MAD2 are not epistatic

The simplest interpretation of the above data is that Bub2p–Dbf2p form a separate branch of the spindle checkpoint. If correct, the phenotypic consequences of *bub2Δ* would not be epistatic to the phenotype of mutation of the genes in the other branch of the pathway. For example, a double mutant between *bub2Δ* and, say, *mad2Δ* would not simply have the phenotype of the latter mutant. Instead, the presence of *bub2Δ* would in some way modify the phenotype of the *mad2Δ* mutation. Several lines of evidence show this to be the case.

A *mad2Δ* strain, YDF37, was crossed with a congenic *bub2Δ* strain, YDF38 (Table I), and four *Mata* spore clones selected, a wild-type, a *mad2Δ*, a *bub2Δ* and the double mutant. These were arrested with α-factor and released in the presence of nocodazole. The ensuing synchronous cell cycles were then followed by flow cytometry (Figure 6A). As expected, the wild-type was blocked with a 2C DNA content due to the metaphase arrest induced by nocodazole. Both the *bub2Δ* and *mad2Δ* single mutants also showed a mitotic delay with a 2C DNA content. However, in contrast to the wild-type strain, this was transient. By 180 min, when the wild-type was still arrested, in both the *mad2Δ* and *bub2Δ* strains the arrest was overcome and cells were proceeding into the next cell cycle. The double mutant presented an entirely different picture. The nocodazole was without noticeable effect and the cells progressed into the next cell cycle with no apparent mitotic delay. By 120 min, the bulk of the population displayed a 4C DNA content and by

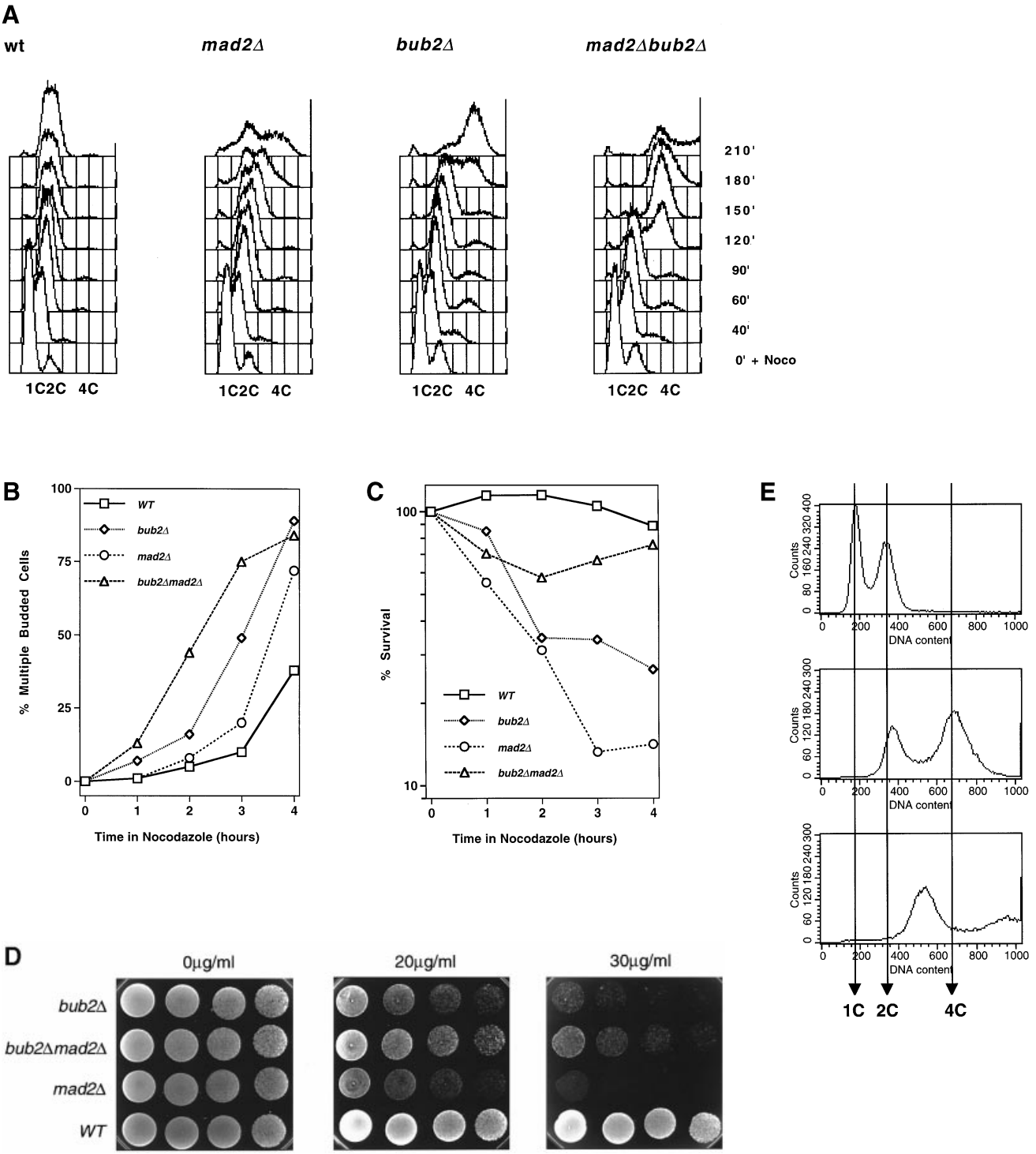

Fig. 6. *bub2Δ* and *mad2Δ* are not epistatic. (**A**) A *bub2Δ* strain, YDF38, was crossed with a congenic *mad2Δ* strain, YDF37, and four *Mat***a** spore clones selected: a wild-type, a *bub2Δ*, a *mad2Δ* and a *bub2Δ mad2Δ*. These were grown to mid-log phase in YEPD and arrested with α-factor for 3 h. They were then released into fresh medium containing 15 μg/ml nocodazole, samples were removed at the intervals shown and prepared for flow cytometry. Note that in the double mutant, an 8C peak occurs that is not shown here. (**B**) Using the same four strains described above, an indentical α-factor synchronization was carried out, once again with release of the cells into 15 μg/ml nocodazole. Cells were harvested, sonicated and the percentage with more than one bud determined microscopically. (**C**) A tetratype tetrad was selected from the cross mentioned above and the viability of the four spore clones was determined in the presence of nocodazole. Exponential cultures were treated with 10 μg/ml nocodazole and, at the intervals shown, samples were removed, sonicated, diluted in saline and spread on YEPD plates in triplicate to measure viability. The data are presented as an average of the three plates. (**D**) Spore clones from the tetratype tetrad used above were grown in YEPD to the same cell number in exponential phase. Cultures were diluted as appropriate, and equal numbers of cells were spotted on YEPD plates containing 0, 20 or 30 μg/ml benomyl. Plates were incubated at 30°C and photographed after 48 h growth. Each spot contains half the cell number of the preceding spot. (**E**) Eight colonies were selected from amongst the survivors of the *bub2Δ mad2Δ* spore clone exposed to nocodazole for 4 h in (C) above. Cultures from these colonies were grown to mid-log phase in YEPD and subjected to FACS analysis. The top panel shows mid-log cells from the *bub2Δ mad2* strain without nocodazole treatment, and the other two panels are cells from two of eight colonies. Note that in the bottom panel a peak of some 6C occurs that is not shown as it is off the linear scale of the graphs.

210 min 8C DNA was evident (not shown). So deletion of both genes appeared to ablate the checkpoint completely. In the case of each single gene deletion, some residual checkpoint function was retained. This can best be explained by Bub2p and Mad2p controlling separate branches of the checkpoint pathway that both impinge upon mitotic arrest.

Another phenotype of spindle checkpoint-defective mutants is repeated budding despite arrest of mitosis (for example, see Hoyt *et al.*, 1991). We therefore examined re-budding in the *bub2Δ mad2Δ* double mutant and compared it with each single mutant (Figure 6B). The double mutant re-budded more rapidly than either the *bub2Δ* or *mad2Δ* strains. This is consistent with the fluorescence-activated cell sorting (FACS) data in Figure 6A and provides further evidence for an additive phenotype of the *bub2Δ* and *mad2Δ* mutations.

In further experiments to explore the question of epistasis, the survival of *bub2Δ*, *mad2Δ* and the double mutant was examined in the presence of low doses of nocodazole. Tetratype tetrads were identified in the cross of YDF37 and YDF38 mentioned above. Cultures from spore clones of a tetratype were incubated in the presence of nocodazole for 4 h and cells were spread on plates without the drug to determine viability (Figure 6C). The *mad2Δ* cells were more sensitive to the drug than *bub2Δ* cells following exposure to 10 μg/ml nocodazole. Surprisingly, the *bub2Δ mad2Δ* double mutant was less sensitive than either of the single mutants. The same result was obtained with a second tetratype tetrad. To confirm this result, sensitivity of the spore clones to benomyl was examined in conventional plating experiments (Figure 6D). Once again, the double mutant is detectably more resistant to the drug than either single mutant, at least in the concentration range of 20–30 μg/ml. At 70 μg/ml, the double mutant is of similar sensitivity to the two single mutants (data not shown).

To gain insight into the basis for the increased resistance of the *bub2Δ mad2Δ* double mutant, eight colonies were selected from survivors of the exposure to 10 μg/ml nocodazole for 4 h. Following flow cytometry, all eight were found to contain at least a 2C DNA content, one being substantially more than 2C (Figure 6C). In contrast, eight wild-type colonies recovered after the same exposure all retained a 1C DNA content (not shown). Investigation of cytokinesis (Hoyt *et al.*, 1991) revealed that this did not occur within the duration of the experiment in Figure 6C (data not shown). Possibly the rapid rounds of S-phase in the double mutant (Figure 6A) in the absence of cytokinesis lead to an increase in ploidy (Figure 6C). Apparent resistance to nocodazole/benomyl might arise from the capacity of cells with increased ploidy to lose chromosomes without invariant loss of viability.

Collectively, the above experiments show clearly that *bub2Δ* and *mad2Δ* are not epistatic. This supports the notion that the two genes lie in separate branches of the spindle checkpoint pathway.

Discussion

Our data suggest that Dbf2p has a very late function in the cell cycle, well after Cdc5p which is required for the late mitotic activation of APC function for Clb degradation (Descombes and Nigg, 1988; Kotani *et al.*, 1998; Shirayama *et al.*, 1998). In fact, the peak in Dbf2p kinase activity coincides with the beginning of Clb kinase inactivation so that Dbf2p could also control APC function in late mitosis. Dbf2p certainly acts downstream of the spindle checkpoint; indeed the Dbf2p-dependent kinase appears to be a target of this checkpoint.

Dbf2p is controlled by the spindle checkpoint

The Dbf2p kinase activity is high during the metaphase arrest at 37°C of *apc2^{ts}* mutants. However, activation of this kinase is prevented following treatment of the cells with the anti-tubulin drug nocodazole, which also arrests cells in metaphase. The drug also prevented activation of Dbf2p in an α-factor-synchronized culture, indicating that the effect is not associated specifically with protracted arrest of the *apc2-8* mutant. Nocodazole activates the spindle checkpoint, so one possibility is that Dbf2p could be a target of the checkpoint. Consistent with this, reciprocal shift experiments confirmed that Dbf2p did act downstream of the checkpoint.

If Dbf2p activation is controlled by the checkpoint, then ablation of the checkpoint by mutation should alleviate inhibition by nocodazole. Deletion of *MAD2* or *BUB3*, components of two complexes that form part of the spindle checkpoint (Figure 7A), was without effect on the inhibition of Dbf2p. However, deletion of *BUB2* resulted in de-regulation of the Dbf2p kinase and its activation in the presence of nocodazole. Dbf2p is, therefore, formally controlled by the spindle checkpoint.

Bub2p and Dbf2p are part of a novel branch to the spindle checkpoint

The spindle checkpoint normally is represented as a linear pathway (Rudner and Murray, 1996; Hardwick, 1998), although several lines of evidence indicate a function for Bub2p distinct from that of the other Mad and Bub proteins. For example, Mad1p and Mad2p are physically associated, as are Bub1p and Bub3p, whilst Bub2p seems not to associate with either complex (Figure 7A). In addition, the cell cycle arrest induced by low doses of nocodazole or the *ctf13^{ts}* mutation, which impairs kinetochore function, depends upon all the *MAD* and *BUB* genes except *BUB2* (Wang and Burke, 1995). Finally, of the checkpoint genes, deletion of *BUB2* alone abolishes the mitotic arrest of *cdc20^{ts}* cells and causes loss of detectable Pds1p at 37°C (Shirayama *et al.*, 1998; Tavormina and Burke, 1998). A possible explanation for the apparently distinct function of Bub2p is provided by our data showing that Bub2p controls Dbf2p. The Mad protein and probably the Bub1p–Bub3p complexes target Cdc20p (see Introduction), and the specific control of Dbf2p by Bub2p argues that they may form a separate branch of the spindle checkpoint pathway. Bub2p–Dbf2p do not, however, form an independent arm of the checkpoint. Rather, they probably form a branch of the existing pathway. Overexpression of Mps1p, a protein kinase located near the top of the pathway (Figure 7A), activates the spindle checkpoint even in the absence of spindle damage (Hardwick *et al.*, 1996). This overexpression alone is sufficient to prevent activation of the Dbf2p kinase. Moreover, Bub2p is known to be required for function of the checkpoint following Mps1p overexpres-

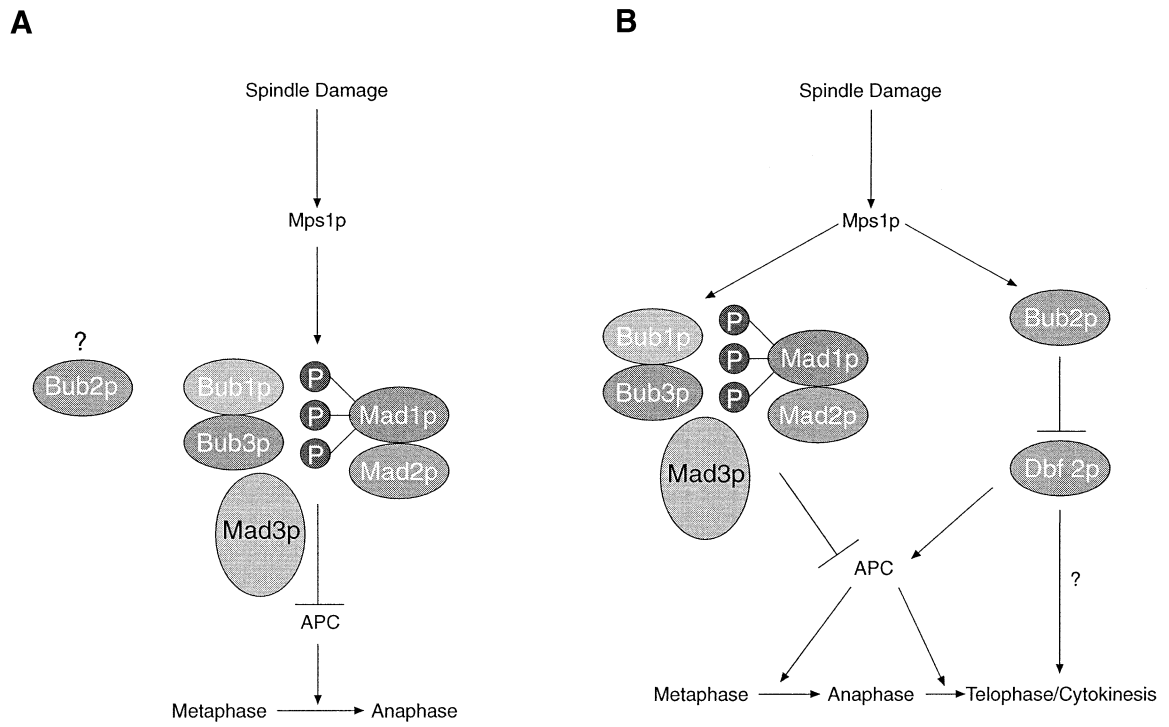

Fig. 7. The budding yeast spindle checkpoint. (**A**) The linear pathway. Bub1p and Bub3p are known to interact as are Mad1p–Mad3p. Both complexes act downstream of Mps1p and target APC, leading to cell cycle arrest at the metaphase–anaphase transition. Bub2p shows few genetic or biochemical interactions with the other Mad/Bub proteins and its function is enigmatic (see text). (**B**) The branched pathway. Bub2p negatively regulates Dbf2p which may regulate APC function in telophase. However, in fission yeast, the Bub2p and Dbf2p homologues regulate cytokinesis. The coordinated regulation of APC and cytokinesis may account for the *dbf2*^{ts} arrest with high cyclin levels.

sion (Hardwick *et al.*, 1996), and we found deletion of *BUB2* to alleviate the repressive effect on Dbf2p of Mps1p overexpression (data not shown). It follows that Dbf2p and Bub2p must, therefore, be part of the checkpoint controlled by Mps1p, but are likely to form a separate branch of the pathway (Figure 7B).

Supporting the notion of a branch in the spindle checkpoint pathway is the additive effect of mutations in the *MAD2* and *BUB2* genes. When treated with nocodazole, cells deleted for either of these genes alone still showed a mitotic delay. However, a markedly different result was obtained with a strain deleted for both genes. This strain showed essentially no mitotic delay in the presence of the drug. This is the expected result if *MAD2* and *BUB2* controlled separate pathways both impinging upon nocodazole-induced mitotic arrest. In addition, the double deleted strain showed rapid re-budding in the presence of nocodazole, again supporting the notion of dual pathways. Finally, the double deleted strain also survived rather better than either single mutant following incubation in low levels of nocodazole. Amongst the *bub2Δ mad2Δ* survivors in this experiment, all eight examined showed an increased ploidy. It is possible that these cells can lose chromosomes in the presence of nocodazole with relative impunity, accounting for their increased viability. All of the above data support a lack of epistasis between *BUB2* and *MAD2*, consistent with Bub2p being part of a second branch to the spindle checkpoint.

Dbf2p and Bub2p are unlikely to be the sole members of this novel branch to the spindle checkpoint pathway. Although we examined deletion of only *MAD2*, *BUB2* and *BUB3*, other *MAD* and *BUB* genes almost certainly are not part of the Bub2p–Dbf2p pathway. In metazoans, Mad2p function depends on Mad1p (Chen *et al.*, 1998) and of course in yeast, Mad1p and Mad2p form a complex. Bub1p–Bub3p also form a complex in yeast and Bub3p is required for the kinetochore localization of Bub1p kinase (Taylor *et al.*, 1998). Moreover, *BUB3* is necessary for the cell cycle effects of *BUB1-5* overexpression (Farr and Hoyt, 1998). Hence, deletion of *MAD2* and *BUB3* effectively ablates the function of all the *MAD* and *BUB* genes, apart from *BUB2*. However, we could find no evidence for a direct physical association between Dbf2p and Bub2p, so at least one other protein is likely to be involved. Supporting this, overexpression of Bub2p does not inhibit the Dbf2p kinase (data not shown), presumably due to the presence of other limiting factors.

One of these other components could be a regulatory factor for Bub2p itself. The homologue of Bub2p in fission yeast is Cdc16p which, together with Byr4p, forms a two-component GTPase-activating system (reviewed in Gould and Simanis, 1997; Furge *et al.*, 1998). Since Bub2p by itself has no known biochemical activity, an analogue of Byr4p is likely to exist in *S.cerevisiae.* Dbf2p also physically interacts with Mob1p (Komarnitsky *et al.*, 1998). This is an essential gene, and conditional mutants in *MOB1* arrest with a cell cycle phenotype identical to that of *dbf2*^{ts} mutants (Luca and Winey, 1998). Dbf2p and Mob1p associate across the entire cell cycle, and this was unaffected by activation of the spindle checkpoint (data not shown). Moreover, Mob1p is associated with active as well as inactive Dbf2p, and there was no evidence that Mob1p levels altered in response to nocodazole treatment or that it could act as an inhibitor of Dbf2p (data not

shown). From these data, Mob1p does not appear to be a component of the Bub2p–Dbf2p branch of the spindle checkpoint, but this needs confirmation. One final protein that might well be part of the Bub2p–Dbf2p pathway is Dbf20p, the Dbf2p homologue. The two genes share some 80% identity and jointly perform an essential late mitotic function (see Introduction). Therefore, Dbf20p could be regulated by Bub2p along with Dbf2p. However, deletion of *DBF20* suggests that its kinase activity is present at only some 10–15% the level of the Dbf2p kinase (Toyn and Johnston, 1994). At present, we have not established an independent assay for Dbf20p, and so whether it too is a target of Bub2p remains obscure.

The biological basis for a second branch to the spindle checkpoint is not clear at present. The *MAD2/BUB1* branch of the pathway suppresses APC function in response to unattached kinetochores (Chen *et al*., 1996; Fang *et al.*, 1998; Taylor *et al.*, 1998, and references therein). The inducer of the *BUB2* pathway is not clear from our present experiments; potentially it might involve the damage induced by high doses of nocodazole (Wang and Burke, 1995). However, it may be significant that Dbf2p physically associates with Mob1p (Komarnitsky *et al.*, 1998) because this, in turn, associates with Mps1p (Luca and Winey, 1998). Mps1p controls duplication of the spindle pole body (SPB) (reviewed in Winey, 1996). Therefore, the association of these proteins might be indicative of Bub2p–Dbf2p monitoring events at the SPB. The intracellular localization of Dbf2p might help to confirm this, but so far we have been unable to detect it.

Regarding the possible role of Dbf2p in the cell cycle, maximal levels of Dbf2p kinase activity occur in anaphase–telophase. This coincides with proteolysis of Clb2p and consequent inactivation of the Clb2p kinase. Supporting the notion that this could be a causal relationship, ubiquitination of cyclin is defective *in vitro* in extracts from *dbf2*^{ts} arrested cells (Jaspersen *et al.*, 1998). Thus Dbf2p may regulate APC function in anaphase or telophase. Interestingly, several APC subunits as well as Cdc20p homologues are phosphorylated in metazoans (King *et al.*, 1995; Kotani *et al.*, 1998; Lorca *et al.*, 1998). A Cdc5p/polo-like protein kinase associates with and phosphorylates some of the APC subunits and has been implicated in activating the APC (Kotani *et al.*, 1998). We were unable to detect any association between Dbf2 and either Cdc23p, an APC component, or Cdc20p (data not shown). Therefore, at present, there is no evidence for direct regulation of the APC by Dbf2p. The branch of the spindle checkpoint pathway that has been characterized of course clearly does block APC-dependent proteolysis, leading to metaphase arrest. Since treatment with nocodazole activates both branches of the pathway, it is difficult to determine whether the Bub2p–Dbf2p pathway could be a specific inhibitor of late mitotic events, specifically Clb degradation. Nocodazole causes gross spindle damage; conceivably, more subtle defects in the spindle apparatus might activate one or other branch of the checkpoint (Wang and Burke, 1995). This might then result in arrest of the cell cycle in metaphase or in telophase, as appropriate.

An alternative possibility is suggested by analysis of late mitotic pathways in fission yeast. *Schizosaccharomyces pombe* has homologues of both Dbf2p (Sid2p) and Bub2p (Cdc16p), as well as other budding yeast late mitotic gene products (Fankhauser *et al.*, 1993; Balasubramanian *et al.*, 1998). In fission yeast, all of these genes control aspects of cytokinesis (reviewed in Gould and Simanis, 1997). If this were true in budding yeast, Bub2p–Dbf2p may regulate cytokinesis rather than Clb2p kinase inactivation. Indeed, the late activation of Dbf2p may support this idea. It is striking that Dbf2p is activated distinctly after Cdc5p which controls Clb degradation, one of the final events of mitosis. The response to a damaged spindle may well require the coordinated inhibition of both APC (cyclin degradation) and cytokinesis. However, to account for the arrest of *dbf2*^{ts} (and other telophase) mutants in late mitosis with high Clb kinase levels (Fitzpatrick *et al.*, 1998), it is then necessary to invoke a regulatory connection between defective cytokinesis and inactivation of mitotic kinase. In other words, the prevention of cytokinesis would in turn have to inhibit Clb degradation in telophase. Whilst plausible, there currently is no evidence for any control of this sort in budding yeast.

This spindle checkpoint is not of course confined to budding yeast, but is highly conserved (Hardwick, 1998). The Mad and Bub proteins occur in *Xenopus* as well as in human cells, where they appear to function in similar checkpoint pathways. In view of this, it is likely that the new branch of the spindle checkpoint that we describe here will also occur in other organisms. A full understanding of this checkpoint is crucial, since defects in the checkpoint can lead to genomic instability and may contribute to oncogenesis (Cahill *et al.*, 1998).

Materials and methods

Strains and media

Relevant yeast strains and their genotypes are shown in Table I. Apart from Figure 1, where a W303-1A-derived strain was used, all other strains were derived from CG378 and CG379, essentially isogenic wild-types. Growth of yeast strains and transformations have been described previously (Kramer *et al.*, 1998). FACS analysis was done using a FACScan Becton Dickinson flow cytometer. For cell cycle arrests, 3.5 μg/ml of α-factor or 15 μg/ml nocodazole were used, unless otherwise stated.

Gene disruptions were carried out by one-step replacement with PCR fragments using YDp plasmids as template (Berben *et al*., 1991). Disruptions were checked by PCR and, where appropriate, benomyl sensitivity.

DNA manipulations

DNA was PCR amplified using *Pfu Taq* polymerase. The *DBF2-3Myc* open reading frame (ORF) was subcloned as a *Bgl*II–*Kpn*I fragment in the pRS304 vector. For integration, pRS304-*DBF2-3MYC* was cut at a unique *Eco*RI site. Alternatively, the *DBF2* ORF was subcloned as a *Sal*I–*Bam*HI fragment in pRL102-*6MYC* (a gift from R.Li, Harvard Medical School, Boston, MA). For integration at the *DBF2* locus, pRL102-*DBF2-6MYC* was cut at a unique *Hin*dIII site. The *CDC5-3HA* ORF was subcloned as a *Bgl*II–*Pst*I fragment in pRS306. For integration at the *CDC5* locus, pRS306-*CDC5-3HA* was cut at a unique *Cla*I site. For overexpression studies, the *MPS1* and *BUB2* ORFs were subcloned as a PCR *Pst*I fragment in pSE402 (pGAL10, 2 μ, *LEU2*) (a gift from S.Enomoto, University of Minnesota, MN), allowing regulated expression at high levels of an N-terminal 6His-T7-tagged Mps1p and Bub2p.

Protein analysis

Protein analyses were performed essentially as described (Kramer *et al.*, 1998), with the following modifications. For Dbf2p and Cdc5p kinase measurement, 0.5 mg of total protein extracts were immunoprecipitated with either anti-Myc (9E10) or anti-HA (12CA5) monoclonal antibodies. Immunoprecipitated Dbf2p and associated histone H1 kinase activity as well as Cdc5p and associated casein kinase were quantified using densitometric scanning and a PhosphorImager (Storm®, Molecular

Dynamics). Dbf2p, Cdc5p, Clb2p, Mps1p and Mad1p were detected by Western blotting using respectively 9E10 (Babco), 12CA5, anti-Clb2p (gift from C.Mann, CEA Saclay), anti-RGS-HIS antibodies (Qiagen) and affinity-purified anti-Mad1p antibodies (gift from K.Hardwick, Edinburgh University, UK).

Overexpression of MPS1

YDF20 containing a galactose-inducible *MPS1* construct were grown overnight in mimimal medium containing 2% sucrose then subcultured in YEP 2% raffinose for 5–6 h. The culture was shifted to 37°C and galactose was added to 4% final concentration. Samples were taken from protein extracts and FACS analysis.

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