# The Bub2-dependent mitotic pathway in yeast acts every cell cycle and regulates cytokinesis

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## SUMMARY

**In eukaryotes an abnormal spindle activates a conserved checkpoint consisting of the *MAD* and *BUB* genes that results in mitotic arrest at metaphase. Recently, we and others identified a novel Bub2-dependent branch to this checkpoint that blocks mitotic exit. This cell-cycle arrest depends upon inhibition of the G-protein Tem1 that appears to be regulated by Bfa1/Bub2, a two-component GTPase-activating protein, and the exchange factor Lte1. Here, we find that Bub2 and Bfa1 physically associate across the entire cell cycle and bind to Tem1 during mitosis and early G1. Bfa1 is multiply phosphorylated in a cell-cycle-dependent manner with the major phosphorylation occurring in mitosis. This Bfa1 phosphorylation is Bub2-dependent. Cdc5, but not Cdc15 or Dbf2, partly controls the phosphorylation of Bfa1 and also Lte1. Following spindle checkpoint activation, the cell cycle phosphorylation of Bfa1 and Lte1 is protracted and some species are accentuated. Thus, the Bub2-dependent pathway is active every cell cycle and the effect of spindle damage is simply to protract its normal function. Indeed, function of the Bub2 pathway is also prolonged during metaphase arrests imposed by means other than checkpoint activation. In metaphase cells Bub2 is crucial to restrain downstream events such as actin ring formation, emphasising the importance of the Bub2 pathway in the regulation of cytokinesis. Our data is consistent with Bub2/Bfa1 being a rate-limiting negative regulator of downstream events during metaphase.**

Key words: Yeast, Bfa1, Bub2, Lte1, Cdc5, Spindle checkpoint, Metaphase

## INTRODUCTION

In eukaryotic cells, inactivation of mitotic kinase, made up of a cyclin-dependent kinase and a B-type cyclin, is required for exit from mitosis. Ubiquitin-mediated proteolysis of the cyclin is principally responsible for the inactivation. An E3 ubiquitin ligase, the anaphase-promoting complex (APC) selects the cyclin for proteolysis. Additional associated regulatory factors are necessary for specificity of target selection, such as Cdc20 at metaphase and Cdh1/Hct1 at anaphase for proteolysis of the mitotic cyclin. Furthermore, in budding yeast, activation of APC requires the Polo-like kinase Cdc5 (Charles et al., 1998) and another group of proteins known as the mitotic exit network (MEN) (reviewed by Morgan, 1999). This includes three protein kinases, Dbf2, Dbf20 and Cdc15, as well as Tem1, a small G-protein, and Lte1, the probable exchange factor for Tem1 (Shirayama et al., 1994). The genes constituting the MEN show many interactions (e.g. Jaspersen et al., 1998; Kitada et al., 1993; Shirayama et al., 1994). This, together with the participation of multiple protein kinases as well as a G-protein, indicates a regulatory network. The late mitotic events controlled by the MEN are likely to include APC activation for its anaphase function (Alexandru et al., 1999; Fesquet et al., 1999; Jaspersen et al., 1998) and also cytokinesis (Frenz et al., 2000 and references therein).

Another regulatory pathway acting in late mitosis and controlling APC function is the spindle assembly checkpoint (SAC) (Amon, 1999; Gardner and Burke, 2000). This is activated by a damaged spindle or unattached kinetochores, and was originally envisaged to be a single pathway resulting in a cell-cycle arrest at metaphase. Recently, a second branch to the SAC was discovered that results in inhibition of mitotic exit (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). This is controlled by Bub2 specifically and the MEN protein kinase Dbf2 was found to be a target of the Bub2 pathway (Fesquet et al., 1999). Taking the data on Bub2 collectively, together with recent work on mitotic exit (Bardin et al., 2000; Pereira et al., 2000; Shou et al., 1999), it seems most likely that Bub2 is controlling the G-protein Tem1. Bub2 has homology to GTPase-activating proteins (GAPs) that inhibit G-protein function. The fission yeast homologue of Bub2, Cdc16, forms a two-component GAP with Byr4 to regulate Spg1, a homologue of Tem1 (Furge et al., 1998). Significantly, Spg1 controls the septum initiation network that is analogous to the MEN (reviewed by Gould and Simanis, 1997; le Goff et al., 1999). Moreover, there is a budding yeast homologue of Byr4, named Bfa1 (Li, 1999), which is part of the SAC, specifically of the Bub2 branch (Alexandru et al., 1999; Li, 1999). All of this points to Bub2/Bfa1 functioning as a two-component GAP to control

Tem1. Lte1, the exchange factor, is also likely to be controlling Tem1. Lte1 is phosphorylated in a cell-cycle-dependent manner and localises to the bud cortex where it is involved in spatial control of MEN activation (Bardin et al., 2000; Pereira et al., 2000).

The mechanism by which mitotic exit is restrained during metaphase arrest is unclear. Tem1 is almost certainly the key to this (Alexandru et al., 1999). Complete inhibition of Tem1 should prevent activation of the MEN and hence mitotic exit. To fully inactivate Tem1 might require stimulation of the Bub2/Bfa1 GAP activity as well as inhibition of Lte1. Accordingly, we have investigated the regulation of these proteins. Both Lte1 and Bfa1, but not Bub2, are phosphorylated in the cell cycle and following SAC activation. Spindle damage, or metaphase arrest caused by other means, simply protracts the normal mitotic phosphorylation of Bfa1. Cdc5 partly controls the cell-cycle phosphorylation of Bfa1, including the phosphorylation seen in metaphase arrest. During metaphase arrest in *bub2Δ* cells, downstream events associated with cytokinesis take place. Our data strongly suggests that the Bub2 pathway acts every cell cycle, and that Bub2 does indeed restrain MEN function in metaphase.

## MATERIALS AND METHODS

### Strains and media

Relevant yeast strain genotypes are indicated in Table 1. All yeast strains were in a CG378 or congenic CG379 background. Growth of yeast strains and transformations have been described previously (Fesquet et al., 1999). Cell-cycle arrests were performed using 3.5 μg/ml α-factor, 0.1 M hydroxyurea or 15 μg/ml nocodazole unless otherwise stated.

The genomic *BUB2* gene was tagged at the C-terminus with a 13MYC epitope by a PCR-based gene integration (Longtine et al., 1998). *BFA1* was tagged at the N-terminus with a 3HA epitope using a PCR-based gene integration technique (Schneider et al., 1995). Tagging was confirmed by PCR. Benomyl sensitivity assays showed that the tagged Bfa1 and Bub2 proteins were fully functional.

### Plasmids and DNA manipulations

The *TEM1* open reading frame was subcloned as a *Bam*HI-*Sma*I PCR fragment into pEMBLYex4 (pGAL, 2 μm, *URA3*) to generate plasmid pSL3, containing 6His-*TEM1* under the control of the inducible GAL promoter.

The Lte1HA3-pTRP1 plasmid was used to tag the endogenous Lte1 protein with 3HA epitopes at the C-terminus. The integrating pTRP1 plasmid carries the sequence for 3 HA epitopes, which can be fused to a protein of interest at the *Not*I site (Mondesert et al., 1997). A *Sal*I/*Not*I fragment spanning the last 500 bases of the *LTE1* gene was cloned into pTRP1. The plasmid was linearised with AflII and integrants selected.

### Over-expression of Tem1

Strain SLY106 containing a galactose-inducible *TEM1* construct (pSL3) was grown overnight in minimal medium containing 2% raffinose, then subcultured in yeast extract-peptone (YEP) 2% raffinose for 4-5 hours. 6His-Tem1 expression was induced by addition of galactose to 2% final concentration. Cells were either harvested 90 minutes after galactose induction, or were induced for 45 minutes prior to a 3 hour incubation period with α-factor, hydroxyurea (HU) or nocodazole, to give a cell-cycle arrest. Samples were taken for protein extraction and nickel precipitation (see below).

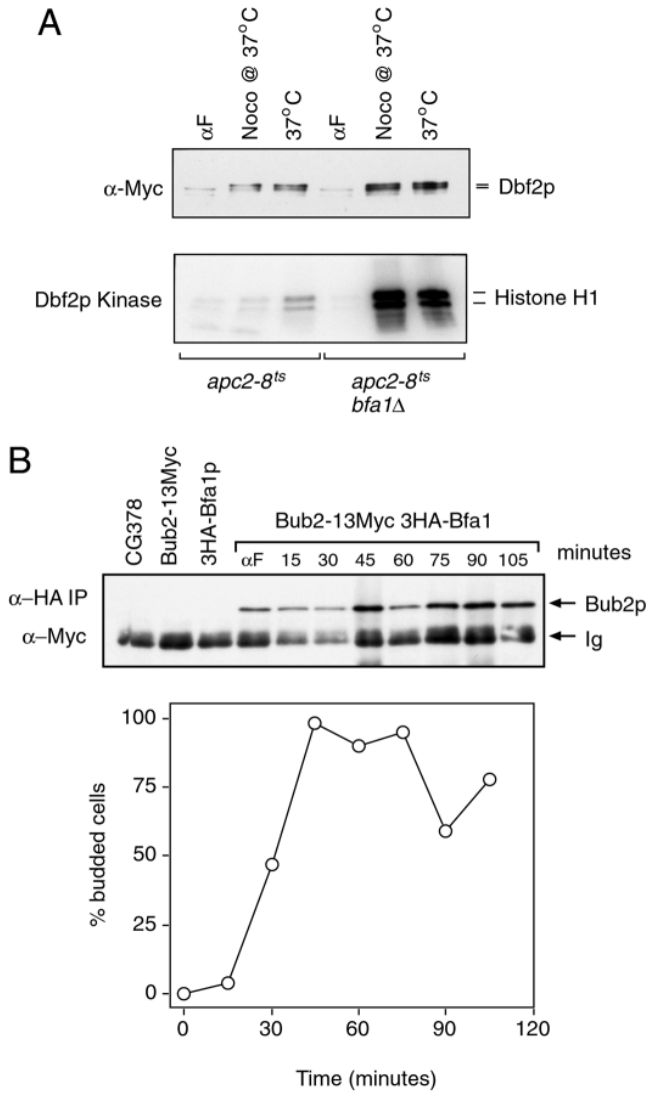


**Fig. 1.** Bfa1 and Bub2 physically associate. (A) Bfa1 negatively regulates Dbf2 kinase activation. Isogenic strains carrying integrated *DBF2-6MYC*, YDF20 *(apc2-8)* and SLY103 *(apc2-8 bfa1Δ)* were grown to mid-log phase at 25°C and arrested with α-factor (αF) or with nocodazole at 37°C (Noco at 37°C) or at 37°C (37°C) for 3 hours. Extracts were immunoprecipitated with anti-Myc (9E10) and Dbf2 kinase assayed (lower panel). The upper part of the kinase assay gel was immunoblotted and probed with 9E10 antibody to control for loading. (B) Bfa1 and Bub2 associate across the cell cycle. Strain SLY106 (*BUB2-13MYC 3HA-BFA1)* was grown to mid-log and synchronised with α-factor. Cells were sampled for protein extraction at the times shown. 300 μg extract was immunoprecipitated using anti-HA (12CA5) antibody, analysed using SDS-PAGE, immunoblotted and probed with 9E10. Lanes 1-3 show an untagged control (CG378) and two singly tagged strains. The Ig band serves as a loading control. The bottom panel shows budding in the synchronised culture.

### Preparation of yeast crude extracts and protein analysis

Cell pellets ($10^7$ cells) were resuspended in 50 μl lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 5 mM EDTA, 1% NP40, 10 mM NaF, 50 mM glycerophosphate, 1 mM DTT, 1mM NaF, 1 mM pNPP, 1 mM glucose-1-phosphate, 10 μg/ml each of the protease inhibitors leupeptin, pepstatin,

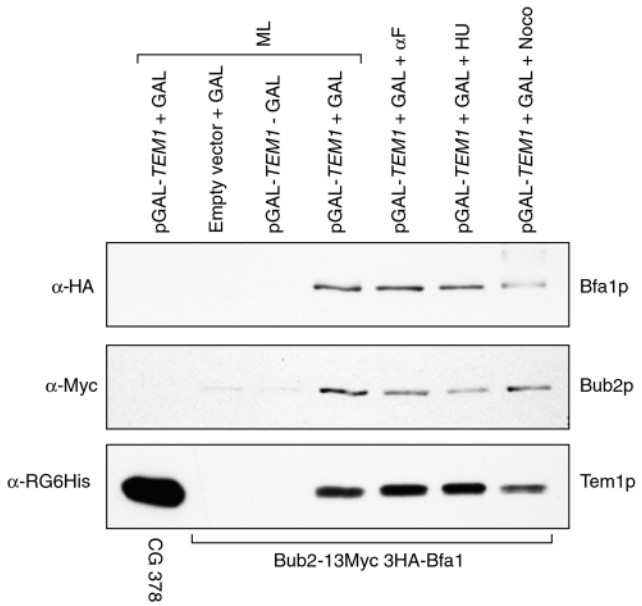


**Fig. 2.** Bfa1 and Bub2 physically associate with Tem1. *pGAL-6HIS-TEM1* was introduced into strain SLY106 (*BUB2-13MYC 3HA-BFA1)* and control strain CG378. Mid-log cells (ML) grown with or without galactose (lanes 1-4) were sampled. The culture of strain SLY106 with galactose was divided and α-factor (αF), HU or nocodazole (Noco) added and incubation continued for 3 hours before sampling. Crude extracts were prepared and incubated with nickel beads. Proteins binding to the beads were eluted and an immunoblot prepared. This was probed with the antibodies shown. Note that strain CG378 is congenic, not isogenic, with strain SLY106, which may account for the high level of Tem1 in lane 1.

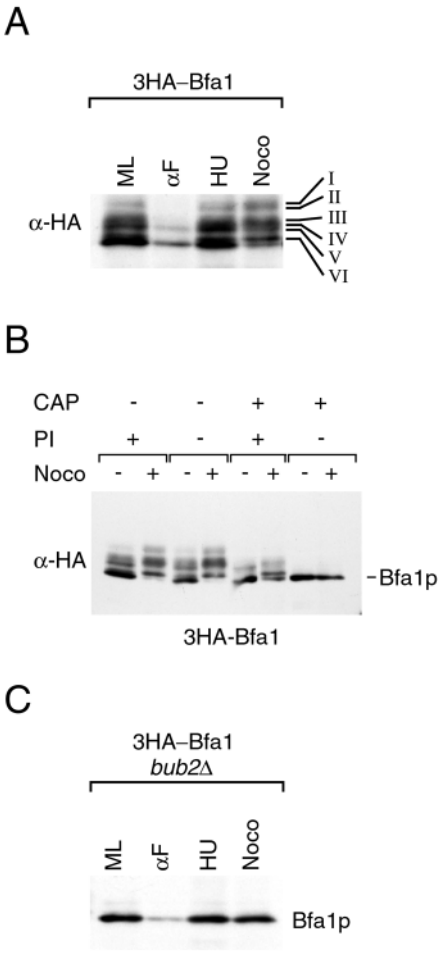


**Fig. 3.** Bfa1 is a phosphoprotein. (A) Mid-log cells of strain SLY105 *(3HA-BFA1)* were harvested or arrested by using α-factor (αF), HU or nocodazole (Noco) for 3 hours before harvesting. Following protein extraction, an immunoblot was prepared and probed with 12CA5. The Roman numerals on the right indicate the various Bfa1 species. (B) Mid-log cells of strain SLY105 were harvested immediately or following treatment with nocodazole for 3 hours. Protein extraction was performed as usual or in lysis buffer without phosphatase inhibitors (+/– PI). 70 μg extract was incubated with calf alkaline phosphatase (CAP) at 30°C for 30 minutes prior to SDS-PAGE and immunoblotting. (C) Phosphorylation of Bfa1 is dependent upon Bub2. Strain SLY111, isogenic to SLY105 but containing *bub2Δ*, was treated as described in (A).

chymotrypsin, aprotinin, antipain and 10 mM PMSF. Cells were disrupted using glass beads. For co-immunoprecipitation experiments, extracts were prepared as described above but with the lysis buffer modified to contain 0.2% NP40.

For immunoblot analysis, 50 μg of protein extract in Laemmli buffer was resolved using SDS-PAGE, transferred to Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and detected using chemiluminescence detection (ECL, Amersham, Little Chalfont, UK) with primary antibodies anti-MYC (9E10), anti-HA (12CA5), anti-Clb2 (gift from C. Mann, CEA Saclay) or anti-RGS-HIS (Qiagen).

For immunoprecipitation, 300 μg of protein extract in 100 μl lysis buffer was immunoprecipitated with 2 μg of anti-MYC or anti-HA antibody on a rotating wheel for 1 hour at 4°C. Protein A/G beads (15 μl) (50% slurry) were added and incubation continued for 30 minutes. The protein A/G beads-immune complex was collected and after washing; co-precipitating proteins were released by boiling in Laemmli buffer.

For the nickel precipitation, 500 μg of protein extract in 100 μl lysis buffer was incubated with 4 μg Ni-NTA beads (Qiagen) for 1 hour at 4°C. The beads were collected and, after washing, co-precipitating proteins were released by boiling in Laemmli buffer.

## RESULTS

### Bfa1 and Bub2 physically associate across the cell cycle

If Bfa1 and Bub2 form a two-component GAP to control Tem1, mutation of either the *BFA1* or *BUB2* genes should have similar effects. Bub2 is needed for down-regulation of Dbf2 kinase activity following SAC activation (Fesquet et al., 1999) and Bfa1 was found to have an identical function (Fig. 1A). That is, strain SLY103 (Dbf2-6Myc *bfa1Δ apc2-8*) displays high Dbf2 kinase activity in the presence of nocodazole. This is consistent with the two proteins having the same function and interacting at some level.

A direct interaction between Bub2 and Bfa1 was established by co-immunoprecipitation of the two proteins (data not shown), confirming recent observations (Pereira et al., 2000). Moreover, we found that Bfa1 and Bub2 associated across the entire cell cycle (Fig. 1B) as well as in the presence of nocodazole (not shown). Tem1 control by Bfa1/Bub2 therefore does not occur by means of transient protein association of the GAP.

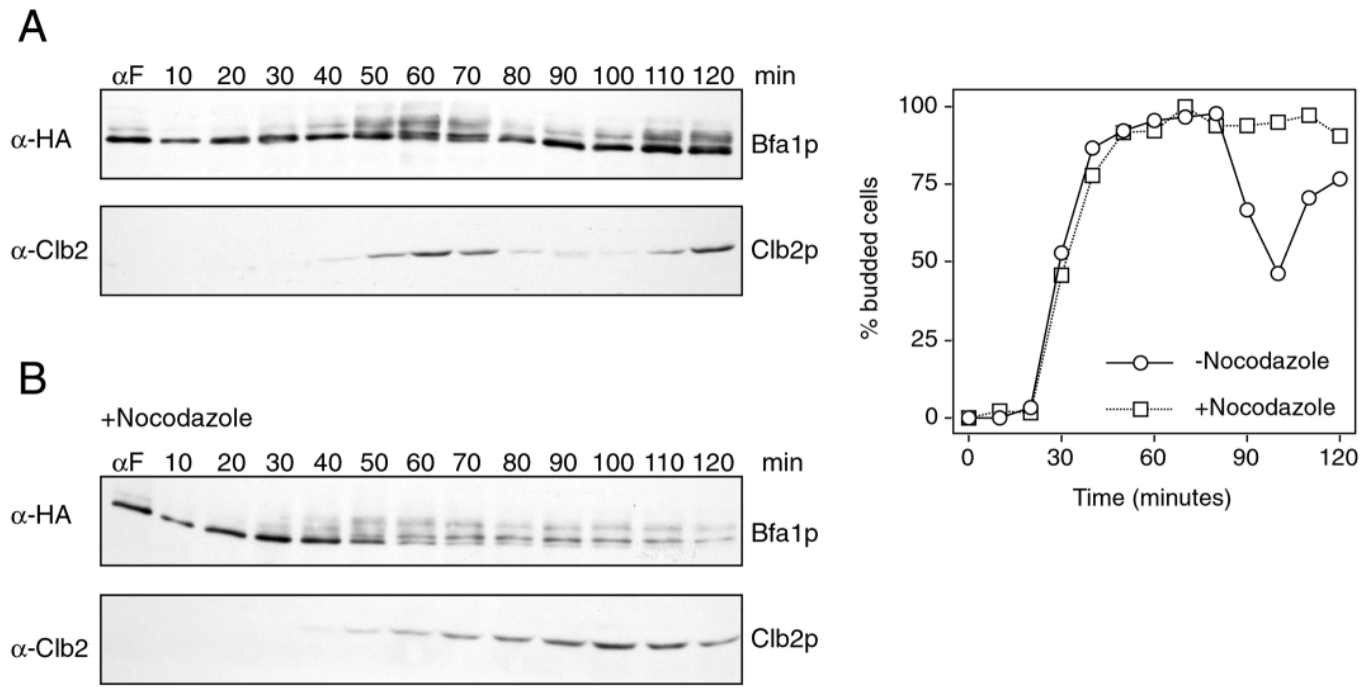


**Fig. 4.** Bfa1 undergoes cell-cycle-dependent phosphorylation that is enhanced following nocodazole treatment. Strain SLY105 (*3HA-BFA1)* was synchronised with α-factor and released into fresh medium in the absence (A) or presence (B) of nocodazole. Samples for protein extraction were taken at the indicated times after release. Extracts were immunoblotted and probed with anti-HA or anti-Clb2 as indicated. The right panel shows budding curves for the two cultures.

## Bfa1 and Bub2 associate with Tem1 during M phase and early G1

The association of Bfa1 and Bub2 with Tem1 was initially examined by expressing GAL-6His-Tem1 in strain SLY106 (Bub2-13Myc 3HA-Bfa1). 6His-Tem1 was precipitated using nickel beads and immunoblotting revealed an interaction with Bfa1 and Bub2 (Fig. 2, lane 4). Surprisingly, the Bfa1/Bub2 association with 6His-Tem1 also occurred in various cell-cycle arrests, including α-factor, hydroxyurea and nocodazole (Fig. 2, lanes 5-7). This data was confirmed by co-immunoprecipitation of Bfa1, Bub2 and Tem1 expressed at normal endogenous levels (data not shown). The complex is therefore likely to persist for much of the cell cycle, unlike most G-proteins and their regulatory factors. Regulation of Tem1 by Bfa1/Bub2 does not therefore happen through transient association. While this manuscript was in preparation, Pereira et al. presented similar data showing the physical interaction of Bfa1 and Bub2 with Tem1 but without addressing the duration of the interaction in the cell cycle (Pereira et al., 2000). Furthermore, we find that in absence of either Bfa1 or Bub2 the remaining protein no longer binds to Tem1 (data not shown).

**Table 1. Yeast strains used in the present study to investigate the mitotic pathway**

| Strain | Genotype | Reference/source |
|---|---|---|
| CG378 | *Mat***a** *ura3 leu2 trp1 ade5* | C. Giroux |
| CG379 | *Matα ura3 leu2 trp1 his7* | C. Giroux |
| YDF20 | *Mat***a** *DBF2*-6MYC::*URA3 apc2Δ*::*HIS7* p*apc2-8** *ura3 leu2 trp1 his7* | Fesquet et al., 1999 |
| YDF21 | *Mat***a** *DBF2*-6MYC::*URA3 apc2Δ*::*HIS7* p*apc2-8 mad2Δ::LEU2 ura3 leu2 trp1 his7* | Fesquet et al.,1999 |
| YDF22 | *Mat***a** *DBF2*-6MYC::*URA3 apc2Δ*::*HIS7* p*apc2-8 bub2Δ::LEU2 ura3 leu2 trp1 his7* | Fesquet et al.,1999 |
| CLF23.11A | *Mat***a** *pGAL.pds1.dbD::LEU2 ura3 his trp1* | This study |
| CLF23.16A | *Mat***a** *pGAL.pds1.dbΔ:: LEU2 bub2Δ:: KAN*$^R$ *ura3 his trp1* | This study |
| SJY121 | *Mat***a** *LTE1*-3HA::*TRP1 ura3 leu2 ade5* | This study |
| SJY122 | Mat**a** *LTE1*-3HA::*TRP1 cdc5/msd2-1::URA3 ura3 leu2 trp1* | This study |
| SLY103 | *Mat***a** *DBF2*-6MYC::*URA3 apc2Δ*::*HIS7* p*apc2-8 bfa1Δ*::*KAN*$^R$ *ura3 leu2 trp1 his7* | This study |
| SLY104 | *Mat***a** *BUB2*-13MYC::*KAN*$^R$ *ura3 leu2 trp1 ade5* | This study |
| SLY105 | *Mat***a** 3HA-*BFA1 ura3 leu2 trp1 ade5* | This study |
| SLY106 | *Mat***a** *BUB2*-13MYC::*KAN*$^R$ 3HA-*BFA1 ura3 leu2 trp1 ade5* | This study |
| SLY109 | *Mat***a** 3HA-*BFA1 cdc5*/*msd2-1*::*URA3 ura3 leu2 trp1 ade5* | This study |
| SLY116 | *Matα* 3HA-*BFA1 apc2Δ*::*HIS7* p*apc2-8 ura3 leu2 trp1* | This study |
| JB5A1-51C | *Mat***a** 3HA-*BFA1 apc2Δ*::*HIS7* p*apc2-8 cdc5/msd2-1::URA3 ura3 leu2 trp1 his7* | This study |
| YLF25 | *Mat***a** *apc2Δ*::*HIS7* p*apc2-8 bub2Δ*::*LEU2 ura3 leu2 trp1 his7* | This study |

*p*apc2-8* is a CEN-based plasmid containing *TRP1* and a temperature-sensitive mutation in *RSI1/APC2*.

### Bfa1 and Lte1 are phosphorylated in a cell-cycle-dependent manner and in response to nocodazole

Immunoblotting showed the stability of both Bub2 and Bfa1 to be constant in the cell cycle (data not shown). Bub2 was also not phosphorylated; however, Bfa1 is clearly a phosphoprotein (Fig. 3A,B). When the *BUB2* gene was deleted, all phosphorylation of Bfa1 was abolished (Fig. 3C). So the heterodimer may be the kinase substrate with the phosphorylations being confined to Bfa1. Alternatively, it was recently shown that Bfa1 localisation to the spindle pole body (SPB) requires Bub2 (Pereira et al., 2000) and this may account for the Bub2-dependence of Bfa1 phosphorylation. The relevant kinase may itself be located on the SPB.

No fewer than six separate Bfa1 phosphorylated species are evident (Fig. 3A, I-VI; see also Figs 4, 6, 7A). There are clear changes in the Bfa1 phosphorylation patterns observed in different cell-cycle arrests (Fig. 3A). The most striking is the almost complete loss of phosphorylation in the α-factor arrest, but there is also a marked change in the nocodazole-treated cells where the phosphorylated band IV is increased slightly (see also Figs 6A, 7A) and band VI is obviously accentuated. The phosphorylated state of Bfa1 therefore changes according to the physiological state of the cells. This was confirmed in a synchronous culture (Fig. 4A), where the Bfa1 phosphorylated species detected corresponded to the six observed in the mid-log sample (Fig. 3A). These phosphorylations start at about 30-40 minutes after bud emergence. However, the bulk phosphorylation becomes evident at 50 minutes coinciding with the appearance of the Clb2 mitotic cyclin near the beginning of mitosis. Much of the Bfa1 phosphorylation is then lost as Clb2 is degraded and it reappears in the next cell cycle.

In a synchronised culture treated with nocodazole, the same range of retarded species was observed (Fig. 4B; see also Fig. 3A), the most prominent differences being an increase in the phosphorylated species VI.

The cell-cycle-dependent phosphorylation of Bfa1 implies that the Bfa1/Bub2 GAP is a normal component of late mitotic control. Consistent with this, the proposed exchange factor for Tem1, Lte1, is also highly phosphorylated in the cell cycle with a similar timing to Bfa1 (Fig. 5A). Similar data regarding Lte1 phosphorylation was recently described by Bardin et al. (Bardin et al., 2000). However, in addition we investigated the phosphorylation profile of Lte1 after SAC activation. When nocodazole was added to half of our synchronous culture, the great majority of Lte1 shifted to the upper phosphorylated species but no additional phosphorylations were observed (Fig. 5B).

This cell-cycle-dependent phosphorylation of Bfa1 and Lte1 shows that they must have a physiological function every cell cycle starting in early mitosis. Treatment of cells with nocodazole, and hence activation of the SAC, protracts the phosphorylation and, presumably, their function. As we are principally interested in Bub2 control, we have concentrated on the regulation of Bub2/Bfa1 for the remainder of this work.

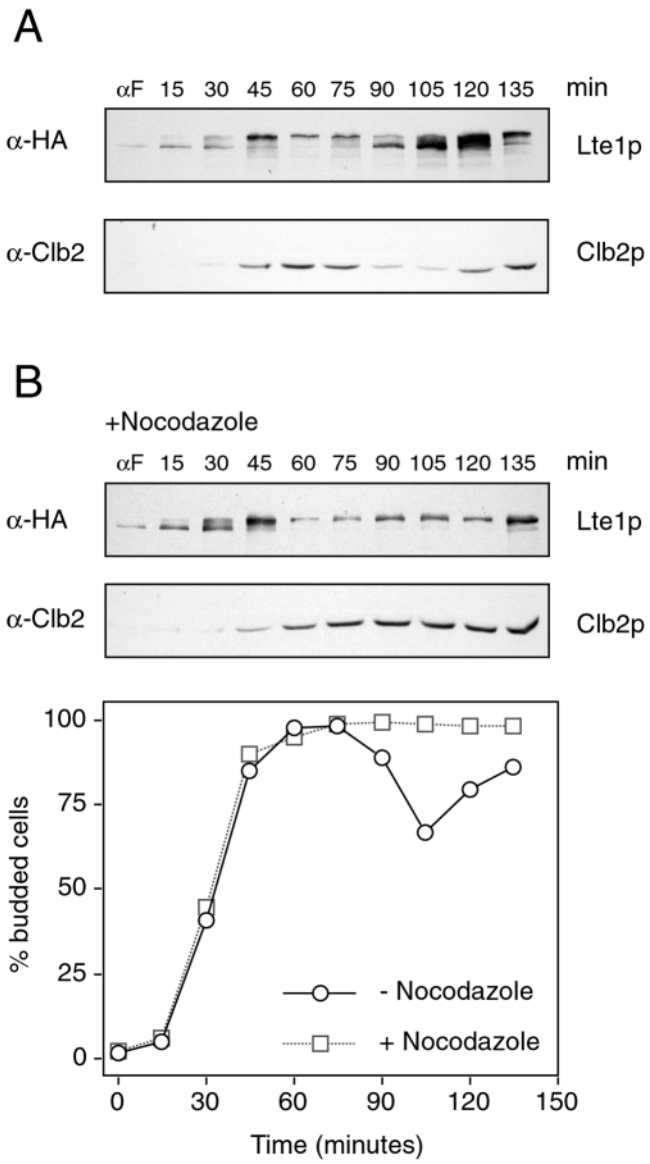


**Fig. 5.** Lte1 undergoes cell-cycle-dependent phosphorylation that is maintained following nocodazole treatment. Strain SJY121 (*LTE1-3HA)* was synchronised with α-factor and released into fresh medium at 25°C in the absence (A) or presence (B) of nocodazole. Samples for protein extraction were taken at the indicated times after release. Immunoblots were analysed with anti-HA or anti-Clb2 as indicated. The lower panel shows budding curves for the two cultures.

### Cdc5 partly controls phosphorylation of Bfa1

Identification of the protein kinases that control phosphorylation of Bfa1 is crucial to understanding regulation of Bub2/Bfa1. Of various mitotic kinases examined, including Mps1, Cdc15 and Dbf2, only the Polo-like kinase Cdc5 affected some of the phosphorylated forms of Bfa1. Transfer of strain SLY 109 (3HA-Bfa1 *cdc5 (msd2-1)*) to 37°C with and without nocodazole revealed significant changes in the Bfa1 phosphorylated species (Fig. 6A; see also Fig. 7A). The two phosphorylated bands IV and VI are slightly reduced in the 37°C cells without nocodazole. Both these bands are clearly accentuated following nococdazole treatment at 25°C and are significantly reduced in level at 37°C indicating that Cdc5 is required for Bfa1 phosphorylation in the presence of nocodazole. In synchronised cultures transferred to an environment at 37°C, bands I and II are absent and are not evident even on longer exposures, so these may also be

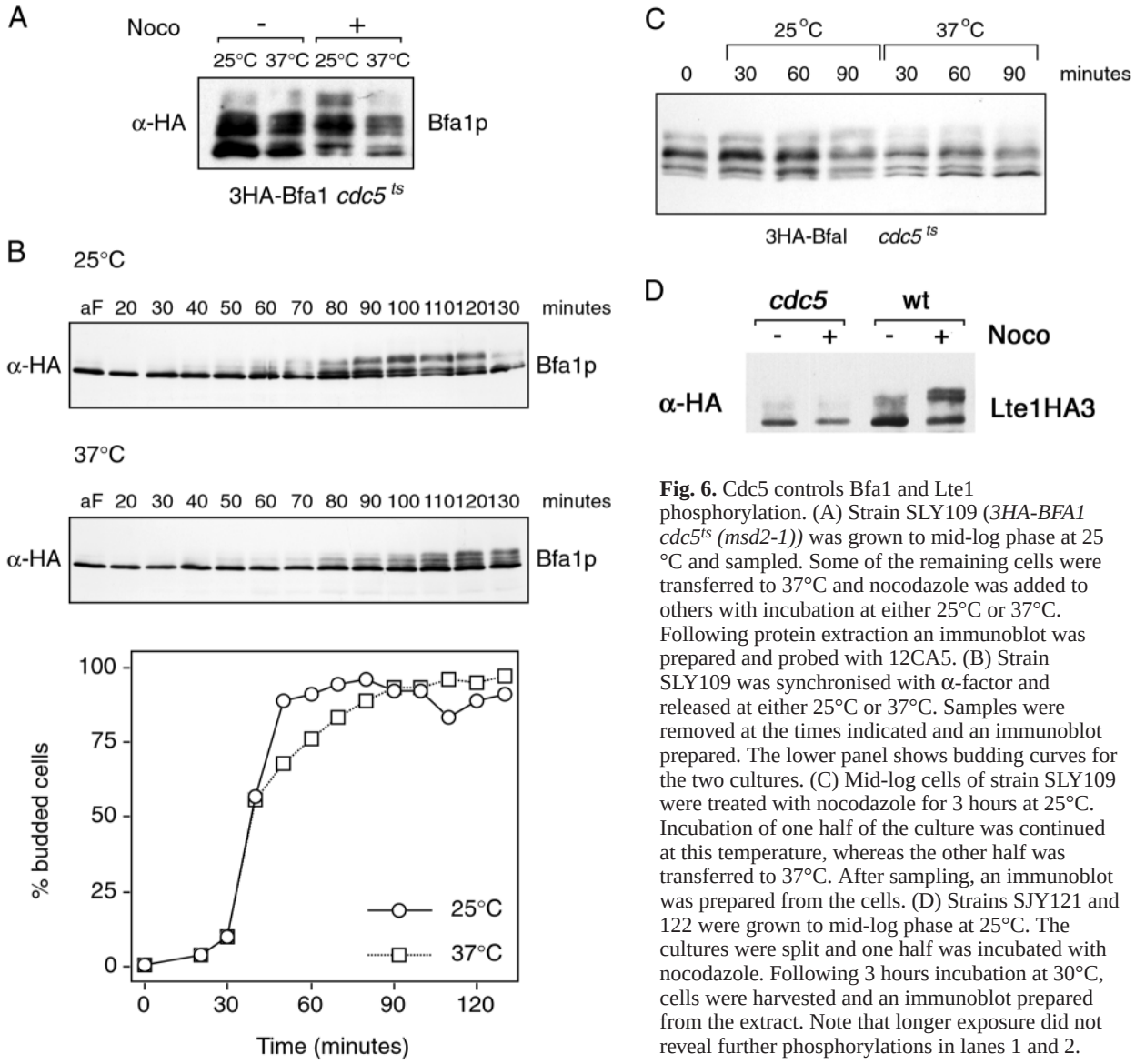


**Fig. 6.** Cdc5 controls Bfa1 and Lte1 phosphorylation. (A) Strain SLY109 (*3HA-BFA1 cdc5$^{ts}$ (msd2-1)*) was grown to mid-log phase at 25 °C and sampled. Some of the remaining cells were transferred to 37°C and nocodazole was added to others with incubation at either 25°C or 37°C. Following protein extraction an immunoblot was prepared and probed with 12CA5. (B) Strain SLY109 was synchronised with α-factor and released at either 25°C or 37°C. Samples were removed at the times indicated and an immunoblot prepared. The lower panel shows budding curves for the two cultures. (C) Mid-log cells of strain SLY109 were treated with nocodazole for 3 hours at 25°C. Incubation of one half of the culture was continued at this temperature, whereas the other half was transferred to 37°C. After sampling, an immunoblot was prepared from the cells. (D) Strains SJY121 and 122 were grown to mid-log phase at 25°C. The cultures were split and one half was incubated with nocodazole. Following 3 hours incubation at 30°C, cells were harvested and an immunoblot prepared from the extract. Note that longer exposure did not reveal further phosphorylations in lanes 1 and 2.

controlled by Cdc5. Bands IV and VI are essentially absent at 37°C (Fig. 6B). Cdc5 therefore clearly controls some of the Bfa1 phosphorylation events.

Interestingly, the hyper-phosphorylated species of Lte1 observed in metaphase-arrested wild-type cells (Fig. 6D, lane 4) are not detectable in nocodazole-treated *cdc5* cells grown at the semi-permissive temperature of 30°C (Fig. 6D, lane 2). It is therefore likely that Cdc5 also regulates the mitotic phosphorylation of Lte1.

Continued function of Cdc5 is necessary for the nocodazole-induced changes in Bfa1 phosphorylation. A culture of strain SLY109 was treated with nocodazole for 3 hours and divided: one half was incubated further at 25°C, while the other half was placed at 37°C. At 25°C the expected enhancement of phosphorylated species IV and VI was observed (Fig. 6C). Significantly, in the cells held at 37°C these two Bfa1-phosphorylated bands progressively diminished in intensity. Thus, during nocodazole-induced metaphase arrest, Cdc5 function is necessary for the continued phosphorylation of Bfa1.

The cell-cycle-dependent phosphorylation of Bfa1 suggests a trivial explanation for the above data. *cdc5* arrest may simply occur at a stage when Bfa1 is experiencing dephosphorylation. A number of experiments rule this out. First, nocodazole induces arrest in metaphase and the effect of *cdc5* was apparent in nocodazole-treated cells (Fig. 6A). Second, in the *cdc5*-synchronised cells incubated at 37°C, species IV does not appear at all, rather than appearing and disappearing. Finally, Bfa1 phosphorylation was examined in cells of *cdc15* and *dbf2* mutants transferred to 37°C. These mutants arrest at about the same point in the cell cycle as *cdc5*. However, at 37°C they do not show any loss of specific Bfa1-phosphorylated bands as we observed with *cdc5*. Instead, the expected bands were detected, which is consistent with the *cdc5* effect being specific and these kinases not affecting Bfa1 phosphorylation (data not shown). Thus Cdc5 clearly controls some Bfa1 phosphorylations.

### Bfa1 phosphorylation in an *apc2-8*-induced metaphase arrest resembles that in a nocodazole-induced metaphase arrest

The phosphorylation of Bfa1 and Lte1 in normal cycling cells

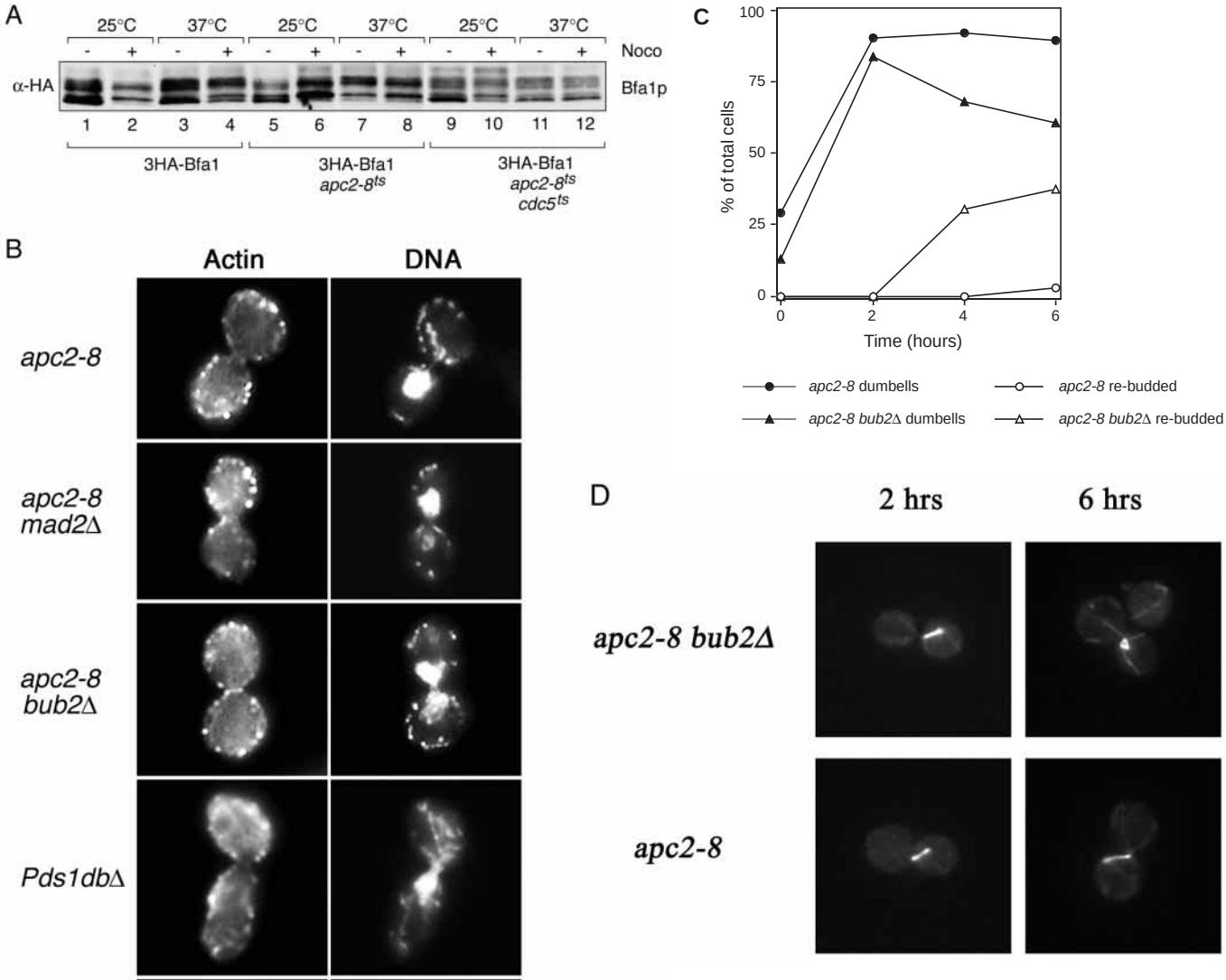


**Fig. 7.** Bub2/Bfa1 act during metaphase arrest caused by means other than spindle damage. (A) Bfa1 shows the same pattern of phosphorylation during *apc2-8* temperature arrest as after nocodazole-induced arrest. Three strains expressing 3HA-Bfa1, a wild type, SLY116 (*apc2-8*) and JB5A1-51C (*apc2-8 cdc5*) were grown to mid-log at 25°C and transferred to 37°C for 3 hours in the presence or absence of nocodazole and an immunoblot prepared. (B) Bub2 restrains actin ring formation in metaphase arrest caused by *apc2-8* temperature shift or overexpression of indestructible Pds1. Mid-log cultures of the *apc2-8* strains were transferred to 37°C for 3 hours. For overexpression of indesructible Pds1, the strains were grown to mid-log phase in YEP sucrose, arrested with α-factor and released into fresh YEP sucrose and galactose added to 2% when all cells had budded. Once large-budded cells had accumulated, samples were removed for microscopy. Cells were stained with DAPI and rhodamine-phalloidin for actin distribution (Frenz et al., 2000). The cells shown above (B) are representative examples of the large-budded cells examined. (C, D) Bub2 restrains mitotic exit in *apc2-8*-arrested cells. Strains containing *TUB1.GFP* in an *apc2-8* or *apc2-8 bub2Δ* background were grown to mid-log phase at 23°C, shifted to 37°C and sampled at 2 hour intervals. Rebudding was assessed microscopically following sonication (C) and the state of the spindles and SPBs examined by fluorescent microscopy (D).

shows that these proteins are physiologically active during every cell cycle rather than just in response to spindle damage. Moreover, there are similarities in the pattern of Bfa1 phosphorylation in the cell cycle and in nocodazole-induced metaphase arrest. This may therefore indicate a protraction of events operating in a normal cell cycle. If correct, metaphase arrest induced by means other than nocodazole treatment should induce similar changes in the pattern of Bfa1 phosphorylation. To examine this, an *apc2-8* strain was transferred to 37°C, which inactivates APC and causes a metaphase arrest like that due to nocodazole treatment.

Cultures of a wild type and strain SLY116 (*apc2-8* 3HA-Bfa1) were grown at 25°C and half were transferred to 37°C. The 25°C and 37°C cultures were further divided and nocodazole was then added to one of each pair of cultures. The wild type showed the expected Bfa1 phosphorylations at mid-

log phase and after nocodazole treatment (Fig. 7A, lanes 1-4). With the *apc2-8* strain the significant result is the similar Bfa1 phosphorylations in the culture incubated at 37°C without nocodazole and those cultures incubated with the drug (compare lane 7 with lanes 2, 4 and 6). So the pattern of Bfa1 phosphorylation is the same whether cells are arrested in metaphase by activation of the SAC or by mutational inactivation of the APC.

Incubation of a *cdc5* mutant at 37°C affected Bfa1-phosphorylated bands IV and VI (Fig. 6). If the *apc2-8* arrest physiologically resembles nocodazole-induced arrest, *cdc5* should affect Bfa1 phosphorylation in a similar way. Strain JB5A1-51C (*apc2-8 cdc5* 3HA-Bfa1) was transferred to 37°C with or without nocodazole and phosphorylated species IV and VI were once again reduced in intensity (Fig. 7A, compare lanes 11 and 7, lanes 12 and 8). The pattern of Bfa1 phosphorylation during metaphase arrest is therefore not specific to nocodazole treatment. Hence Bub2/Bfa1 is part of an intrinsic mitotic control that operates during metaphase.

Bub2 and Bfa1 would be expected to have a negative effect on events controlled by Tem1, as we have demonstrated for Dbf2 kinase activity (Fesquet et al., 1999; Fig. 1A). If the *apc2-8* arrest is physiologically equivalent to that induced by nocodazole, deletion of *BUB2* should similarly permit downstream cell-cycle events prevented by the protracted activation of the Bub2/Bfa1-dependent pathway. In the *apc2-8*-induced metaphase arrest, a normal cortical distribution of actin patches occurred without formation of an actin ring at the bud neck (Fig. 7B, top panel). However, when *BUB2* was deleted from this strain, the actin ring was formed in metaphase during *apc2-8* incubation at 37°C. Deletion of *MAD2* from the *apc2-8* strain was without effect. This result was not simply due to secondary effects caused by mutational inactivation of APC. When metaphase arrest was achieved by over-expressing indestructible Pds1 (Cohen-Fix et al., 1996), the same result was obtained. That is, following Pds1Δdb over-expression in a wild type, actin patches were cortical as expected for a metaphase arrest, whereas overexpression in a *bub2Δ* strain resulted in the inappropriate formation of an actin ring (Fig. 7B, bottom two panels). Thus the Bub2-dependent pathway is activated during metaphase to inhibit downstream events. The formation of the actin ring, which is one of the initial events of cytokinesis, is probably a reflection of Tem1 control of cytokinesis through regulation of the MEN (Frenz et al., 2000).

To explore further the events controlled by Bub2, in a similar experiment we monitored the localisation of Dbf2-GFP in *apc2-8, apc2-8 bub2Δ* and *apc2-8 mad2Δ* strains. Dbf2 localised solely to the SPBs (data not shown) and no bud neck staining was evident (Frenz et al., 2000). Thus Bub2 does not control localisation of Dbf2 and, presumably, other MEN proteins. However, upon protracted incubation of cells at 37°C, the *apc2-8 bub2Δ* strain showed some 40% rebudding (Fig. 7C). Moreover, SPB duplication was also observed in these cells (Fig. 7D). Because these events do not occur in the *apc2-8* strain, deletion of *BUB2* must allow mitotic exit in a proportion of the *apc2-8 bub2Δ*-arrested cells. Presumably, Cdc14 is released leading to Cdc28 inactivation by Sic1 (Shou et al., 1999). However, Cdc14 release is transient and we were unable to observe this. Of particular interest is the absence of cytokinesis in the rebudded cells. Although Bub2 controls some aspects of cytokinesis, such as actin ring formation, additional controls are clearly necessary for later events, including relocalisation of MEN proteins such as Dbf2.

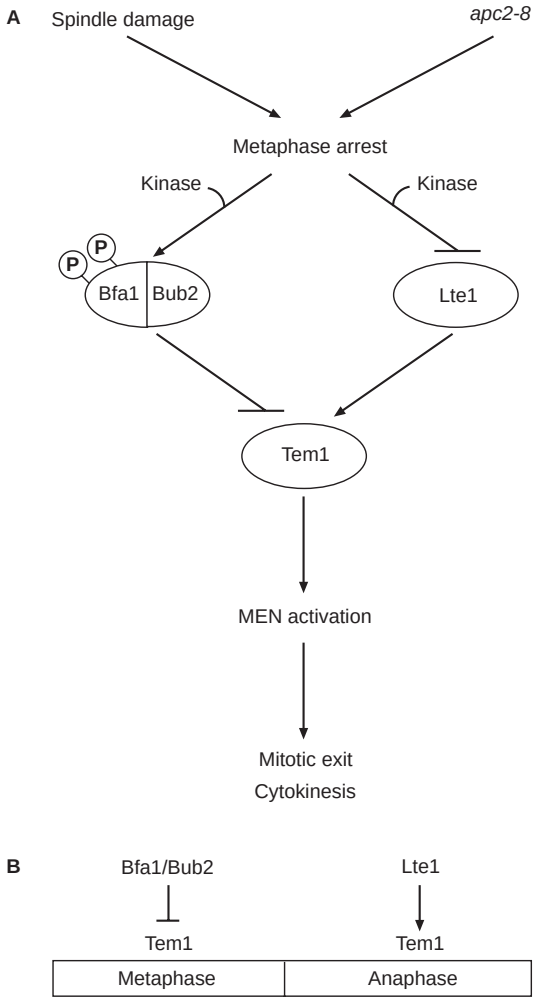


**Fig. 8.** Metaphase arrest activates Bfa1/Bub2 to inhibit Tem1 and shut down the MEN. (A) Metaphase arrest induced by SAC activation or the *apc2-8* mutation leads to a presumed activating phosphorylation of Bfa1. This Bfa1 phosphorylation, together with phosphorylation of Lte1, inactivate Tem1 to block activation of the MEN (including Dbf2) and cytokinesis (Frenz et al., 2000). (B) Bub2/Bfa1 control of Tem1 is rate limiting in metaphase to block MEN activation. Lte1 positive control of Tem1 occurs in anaphase and promotes mitotic exit.

## DISCUSSION

Our data support the notion that Bfa1 is a regulatory subunit for Bub2. The two proteins physically interact but Bfa1 alone shows cell-cycle-dependent phosphorylations. Because Bub2 has sequence homology to GAP proteins, Bfa1 presumably modulates this GAP activity. Consistent with Bfa1/Bub2 regulating Tem1 to block MEN function, both proteins physically associate with it, presumably leading to inactivation of Tem1 as appropriate. This interpretation is entirely consistent with the comparable system in fission yeast. The Bfa1/Bub2 homologues Byr4 and Cdc16 are known to form a

two-component GAP (Furge et al., 1998). Cdc16 contains GAP homology and, like Bfa1, Byr4 shows electrophoretic mobility shifts, although it was not shown to be a phosphoprotein (Song et al., 1996). Moreover, Cdc16 and Byr4 also interact directly with the G-protein Spg1 (Gardner and Burke, 2000; Jwa et al., 1998; Song et al., 2000). Finally, Spg1 is a key regulator of the septum initiation network, which is analogous to the MEN (see Introduction).

A GAP activity, of course, acts negatively and we find that deletion of Bub2 or Bfa1 alone is sufficient to allow downstream events during metaphase arrest: Dbf2 kinase activation (Fig. 1A; Fesquet et al., 1999), actin ring formation and ultimately mitotic exit. Regarding actin ring formation, in *apc2-8*- induced metaphase arrest actin patches are cortical but in an *apc2-8 bub2Δ* double mutant, the absence of active Bfa1/Bub2 permits actin ring formation at the bud neck. This was not an aberration due to mutational inactivation of APC as the same effect was observed in cells over-expressing an indestructible Pds1. This is a particularly striking observation as actin ring formation is one of the initial steps in cytokinesis. Its occurrence during metaphase arrest in *bub2Δ* cells shows clearly that Bub2 is a regulator of cytokinesis. Interestingly, in *apc2-8 bub2Δ*-arrested cells Dbf2 does not relocate from the SPBs to the bud neck and in those cells in which mitotic exit (rebudding) occurs, cytokinesis is not completed. Hence, additional controls are required for the later steps of cytokinesis.

A critical question with regard to Bfa1/Bub2, and Lte1, is how their activation is regulated. Control of Bfa1/Bub2 does not occur through their transient association with one another or with Tem1. The interaction with Tem1 is long-lived and appears to persist at least for much of mitosis and into G1 (α-factor arrest). This is unusual for a GAP activity that normally binds only briefly to its substrate. At present, the only significant regulatory feature detected for Bfa1/Bub2 and Lte1 is phosphorylation. To characterise this fully requires mapping the individual sites and assessing their relevance in an in vitro GTPase assay for Tem1, which is clearly beyond the scope of this study.

The simplest interpretation of the Bfa1 phosphorylation is that it is important for activation of the GAP activity and hence inactivation of Tem1 (Fig. 8A). In a normal cell cycle this would tend to inactivate Tem1 for much of mitosis presumably to inhibit mitotic exit and cytokinesis. Then at the appropriate time, Tem1 would be activated at least partly by dephosphorylation of Bfa1. By contrast, Lte1 phosphorylation would be inactivating and its dephosphorylation late in mitosis would stimulate the exchange factor contributing further to the rapid activation of Tem1 in anaphase.

During metaphase arrest caused by either nocodazole treatment or *apc2-8* arrest at 37°C, the same Bfa1-phosphorylated species are apparent as seen in the cell cycle. However, there are changes in the ratio of various species and, importantly, these are the same in both types of arrest. The Bfa1 phosphorylation changes are therefore not specific to SAC activation but may occur in any metaphase arrest. Most likely, metaphase arrests simply protract the normal physiological role of Bfa1/Bub2 in order to shut down Tem1 and block cytokinesis.

The phosphorylation of Bfa1 and Lte1 suggests that there may be intracellular signalling pathways impinging upon these Tem1 regulatory proteins (Fig. 8A). The only protein kinase we detected with any effect on the pattern of Bfa1 phosphorylation is Cdc5. Incubation of a *cdc5* temperature-sensitive mutant at 37°C resulted in changes to the pattern of Bfa1 phosphorylation. Continued Cdc5 activity is also necessary for maintaining the nocodazole-induced Bfa1 phosphorylation changes. However, whether Cdc5 directly phosphorylates Bfa1 in vivo is not yet clear. In in vitro kinase assays, full length Bfa1 was a poor substrate for Cdc5, although an N-terminal fragment of the protein was a particularly good substrate (data not shown). In addition, we found Lte1 phosphorylation to be under Cdc5 control. Unfortunately Cdc5 phosphorylation sites have not been characterised. Further work is therefore clearly necessary, once again entailing definition of Bfa1 and Lte1 phosphorylation sites, to establish whether they are direct Cdc5 substrates. Interestingly, Plo1, the fission yeast homologue of Cdc5, has recently been shown to act upstream of the septation initiation network, which is analogous to the MEN (Tanaka et al., 2001).

One final implication of our data concerns the intracellular localisation of Tem1. In metaphase arrested cells deletion of Bub2 or Bfa1 is sufficient to allow downstream events to occur (see above). In such cells Tem1 is located on SPBs (Pereira et al., 2000; S.J., unpublished), which in metaphase mostly lie in the mother cell. Thus Bfa1/Bub2 are clearly the rate-limiting controls operating on Tem1 to prevent mitotic exit and cytokinesis in metaphase. This conclusion is also implicit in the data of Bardin et al. (2000) who showed that Bub2 prevents mitotic exit when nuclear division takes place in the mother cell. In metaphase, Lte1 is confined to the daughter cell (Bardin et al., 2000; Pereira et al., 2000) so upon movement of one SPB into the daughter, Tem1 encounters Lte1 for the first time, which presumably triggers Tem1 activation. Formally, Bfa1/Bub2 are therefore negative regulators of Tem1 in metaphase and prevent downstream events (Fig. 8B). By contrast, Lte1 may be a positive activator of Tem1 functioning in anaphase to promote mitotic exit. Hence, for much of mitosis, Bfa1/Bub2 and Lte1 impose their control in different mitotic phases and are temporally distinct. When Tem1 enters the daughter, the balance between Lte1 and Bub2 control of Tem1 must shift in favour of Lte1 as part of the commitment to mitotic exit. In agreement with these antagonistic effects on Tem1 activity, we find that deletion of Bfa1 or Bub2 is sufficient to rescue the cold-sensitivity of *lte1Δ* mutants (data not shown), consistent with the model in Fig. 8.

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## REFERENCES

**Alexandru, G., Zachariae, W., Schleiffer, A. and Nasmyth, K.** (1999). Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. *EMBO J.* **18,** 2707-2721.

**Amon, A**. (1999). The spindle checkpoint. *Curr. Opin. Gen.. Dev.* **9,** 69-75.

**Bardin, A. J., Visintin, R. and Amon, A**. (2000). A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell* **102,** 21-31.

**Charles, J. F., Jaspersen, S. L., Tinker-Kulberg, R. L., Hwang, L., Szidon, A. and Morgan, D. O.** (1998). The *polo*-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*. *Curr. Biol*. **8**, 497-507.

**Cohen-Fix, O., Peters, J. M., Kirschner, M. W. and Koshland, D**. (1996). Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* **24,** 3081-3093.

**Fesquet, D., Fitzpatrick,P. J., Johnson, A. L., Kramer, K. M., Toyn, J. H. and Johnston, L. H.** (1999). A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast. *EMBO J.* **18**, 2424-2434.

**Fraschini, R., Formenti, E., Lucchini, G. and Piatti, S.** (1999). Budding yeast Bub2 is localised at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. *J. Cell Biol.* **145**, 979-991.

**Frenz, L. M., Lee, S. E., Fesquet, D. and Johnston, L. H**. (2000). The budding yeast Dbf2 protein kinase localises to the centrosome and moves to the bud neck in late mitosis *J. Cell Sci.* **113,** 3399-3408.

**Furge, K. A., Wong, K., Armstrong, J., Balasubramanian, M. and Albright, C. F.** (1998). Byr4 and Cdc16 form a two-component GTPase-activating protein for the Spg1 GTPase that controls septation in fission yeast. *Curr. Biol.* **8**, 947-954.

**Gardner, R. D and Burke, D. J.** (2000). The spindle checkpoint: two transitions, two pathways. *Trends Cell Biol.* **10**, 154-158.

**Le Goff, X., Woollard, A. and Simanis, V.** (1999). Analysis of the cps1 gene provides evidence for a septation checkpoint in Schizosaccharomyces pombe. *Mol. Gen. Genet.* **262**,163-172.

**Gould, K. L. and Simanis, V**. (1997). The control of septum formation in fission yeast. *Genes Dev.* **11,** 2939-2951.

**Jaspersen, A. L., Charles, J. F., Tinker-Kulberg, R. L. and Morgan, D. O**. (1998). A late mitotic regulatory network controlling cyclin destruction in *Saccharomyces cerevisiae. Mol. Biol. Cell* **9**, 2803-2817.

**Jwa, M. and Song, K**. (1998). Byr4, a dosage-dependent regulator of cytokinesis in *S. pombe*, interacts with a possible small GTPase pathway including Spg1 and Cdc16. *Mol. Cells* **8**, 240-245.

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

**Li, R**. (1999). Bifurcation of the mitotic checkpoint pathway in budding yeast. *Proc.Natl. Acad. Sci. USA* **96**, 4989-4994.

**Longtine, M. S., McKenzie, A., III, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P. and Pringle, J. R.** (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* **14**, 953-961.

**Mondesert, G., Clarke, D. J. and Reed, S. I.** (1997). Identification of genes controlling growth polarity of the budding yeast *Saccharomyces cerevisiae*: a possible role of N-glycosylation and involvement of the exocyst complex. *Genetics* **147**, 421-434.

**Morgan, D. O.** (1999). Regulation of the APC and the exit from mitosis. *Nature Cell Biol.* **2**, E47-E53.

**Pereira, G., Höfken, T., Grindlay, J., Manson, C. and Schiebel E.** (2000). The BUB2p spindle checkpoint links nuclear migration with mitotic exit. *Mol. Cell* **6**, 1-10.

**Schneider, B. L., Seufert, W., Steiner, B., Yang, Q. H. and Futcher, B.** (1995). Use of polymerase chain reaction epitope tagging for protein tagging in *Saccharomyces cerevisiae. Yeast* **11**, 1265-1274.

**Shirayama, M., Matsui, Y., Tanaka, K. and Toh-e, A**. (1994). Isolation of a CDC25 family gene, MSI2/LTE1, as a multicopy suppressor of *ira1*. *Yeast* **4**, 451-461.

**Shou, W., Seol, J-H., Schechenko, A., Baskerville, C., Moazed, D., Chen, S., Jang, J., Shevchenko, A., Charbonneau, H. and Deshaies, R. J.** (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from the nucleolar RENT complex. *Cell* **97**, 233-244.

**Song, K., Mach, K. E., Chen, C. Y., Reynolds, T. and Albright C. F.** (1996). A novel suppressor of *ras1* in fission yeast, *byr4*, is a dosage-dependent inhibitor of cytokinesis. *J. Cell Biol*. **133**, 1307-1319.

**Song, S., Grenfell, T. Z., Garfield, S., Erikson, R. L. and Lee, K. S.** (2000). Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. *Mol. Cell Biol.* **20,** 286-298.

**Tanaka, K., Petersen, J., MacIver, F., Mulvihill, D. P., Glover, D. M. and Hagan, I. M.** (2001). The role of Plo1 kinase in mitotic commitment and septatmion in *Schizosaccharomyces pombe*. *EMBO J.* **20,** 1259-1270.