Disappearance of the budding yeast Bub2–Bfa1 complex from the mother-bound spindle pole contributes to mitotic exit

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Budding yeast spindle position checkpoint is engaged by misoriented spindles and prevents mitotic exit by inhibiting the G protein Tem1 through the GTPase-activating protein (GAP) Bub2/Bfa1. Bub2 and Bfa1 are found on both duplicated spindle pole bodies until anaphase onset, when they disappear from the mother-bound spindle pole under unperturbed conditions. In contrast, when spindles are misoriented they remain symmetrically localized at both SPBs. Thus, symmetric localization of Bub2/Bfa1 might lead to inhibition of Tem1, which is also present at SPBs. Consistent with this hypothesis, we show that a Bub2 version symmetrically localized on both SPBs throughout the cell cycle prevents mitotic exit in mutant backgrounds that partially impair it. This effect is Bfa1 dependent and can be suppressed by high Tem1 levels. Bub2 removal from the mother-bound SPB requires its GAP activity, which in contrast appears to be dispensable for Tem1 inhibition. Moreover, it correlates with the passage of one spindle pole through the bud neck because it needs septin ring formation and bud neck kinases.

Introduction

At the end of mitosis, after chromosome segregation, eukaryotic cells must inactivate the cyclin B–dependent kinases that lead them into and through mitosis. This inactivation is necessary for spindle disassembly, cytokinesis, and entry into a new round of DNA replication in the subsequent cell cycle. Critical to this process is cyclin B proteolysis triggered by the anaphase-promoting complex/cyclosome (Peters, 2002). Inactivation of mitotic Cdks in budding yeast is driven by activation of a complex signal transduction cascade, called the mitotic exit network (MEN), which is required for mitotic exit and cytokinesis. The MEN comprises several factors, including a small G protein of the Ras family (Tem1), its activator (Lte1), several protein kinases and associated factors (namely Cdc5, Cdc15, Mob1/Dbf2, Dbf20, and Cla4), and a scaffold protein (Nud1). The latter acts as a platform for many MEN components at the microtubule organizing center or spindle pole body (SPB; Simanis, 2003; Seshan and Amon, 2004). A similarly organized pathway, the septation initiation network, drives cytokinesis in fission yeast (Simanis, 2003), and homologues of several MEN and septation initiation network factors can be found in multicellular eukaryotes. The ultimate effector of MEN signaling is the Cdc14 protein phosphatase, which on one side can directly reverse Cdk phosphorylation events (Gray et al., 2003) and on the other promotes inactivation of cyclin B–dependent kinases by triggering anaphase-promoting complex/cyclosome–dependent cyclin proteolysis and accumulation of their specific inhibitor Sic1 (for review see Stegmeier and Amon, 2004). Though completed by the MEN in telophase, Cdc14 activation is already initiated during anaphase by the action of the Cdc14 early anaphase release (FEAR) pathway, which includes the polo kinase Cdc5 and the separase Esp1 (Stegmeier et al., 2002).

To ensure balanced chromosome partitioning, inactivation of mitotic Cdks must not be initiated before telophase, i.e., before sister chromatid segregation is complete. This issue is vital for organisms like budding yeast, which define the cleavage plane early in the cell cycle and before bipolar spindle formation. In fact, in *Saccharomyces cerevisiae*, the constriction between mother and daughter cells (bud neck) appears at the G1–S transition concomitantly with bud emergence and dictates where cytokinesis will later take place. The spindle positioning checkpoint is responsible for delaying cytokinesis until the spindle enters the bud. The Bub2/Bfa1 GTPase-activating protein (GAP) plays a key role in this process by keeping Tem1 inactive until the spindle is properly oriented, thus inhibiting MEN

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Abbreviations used in this paper: FEAR, Cdc14 early anaphase release; GAP, GTPase-activating protein; GDP, guanosine 5′-diphosphate; MBP, maltose binding protein; MEN, mitotic exit network; SPB, spindle pole body.

The online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 172, No. 3, January 30, 2006 335–346
http://www.jcb.org/cgi/doi/10.1083/jcb.200507162

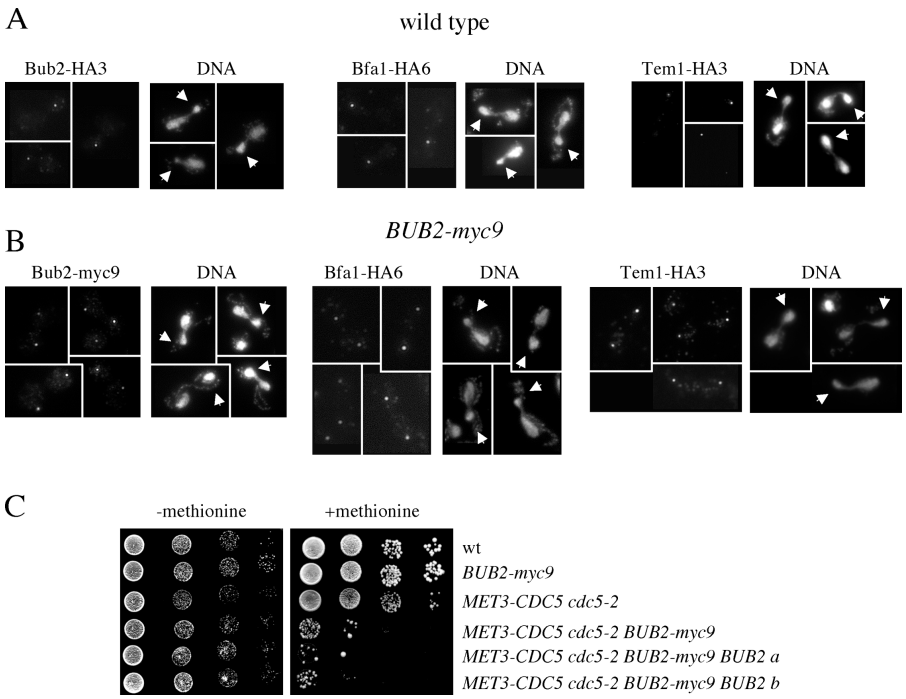


Figure 1. **Effects of symmetrically localized myc-tagged Bub2 on Bfa1 and Tem1 localization and *cdc5-2* cell viability.** (A) Exponentially growing cells expressing Bub2-HA3 (ySP3866), Bfa1-HA6 (ySP2035), or Tem1-HA3 (ySP3641) were stained by indirect immunofluorescence with anti-HA antibodies and mounted with DAPI to stain DNA. (B) Cells expressing Bub2-myc9 alone (ySP710) or in combination with Bfa1-HA6 (ySP5087) or Tem1-HA3 (ySP4625) were treated as in A, using anti-myc antibodies to detect Bub2-myc9. Only anaphase cells were photographed. The arrows point to the bud. (C) Serial dilutions of logarithmically growing cultures of wild-type (ySP41), *BUB2-myc9* (ySP710), *MET3-CDC5 cdc5-2* (ySP3426), and *MET3-CDC5 cdc5-2 BUB2-myc9* (ySP3442) cells, either untransformed or carrying one extra copy of *BUB2* integrated at the *URA3* locus (*MET3-CDC5 cdc5-2 BUB2-myc9 BUB2* a and b, ySP3743, and ySP3744), were spotted on –Met (*MET3* promoter on) or +Met (*MET3* promoter off) plates and incubated for 2 d at 25°C.

activation (for review see Stegmeier and Amon, 2004). Bub2/Bfa1 is found on both SPBs soon after SPB duplication but only on the SPB directed into the bud, along with Tem1, from the onset of anaphase until the end of mitosis. This observation, along with the finding that the Tem1 activator Lte1 is localized specifically in the bud, led to the proposition that MEN activation is triggered by an encounter between Tem1 sitting on the bud-directed SPB and Lte1, thus coupling properly oriented spindle elongation with mitotic exit (Bardin et al., 2000; Pereira et al., 2000). However, Lte1 is required for mitotic exit only at low temperatures (Shirayama et al., 1994) and is dispensable for inappropriate mitotic exit of mutants with spindle positioning defects (Adames et al., 2001). Thus, other mechanisms, such as inactivation of the GAP Bub2/Bfa1, must ensure the timely activation of Tem1. Inhibitory phosphorylation of Bfa1 by the Polo kinase Cdc5, although not essential, clearly contributes to this task (Hu et al., 2001). Unlike Bub2/Bfa1, Tem1 is also present on the mother-bound SPB from the time of bipolar spindle formation to telophase. This suggests that disappearance of Bub2/Bfa1 from that SPB at the onset of anaphase might be important for proper MEN activation. In agreement with this hypothesis, activation of the spindle position checkpoint by either microtubule depolymerization or mutations impairing spindle orientation preserves Bub2/Bfa1 on both SPBs (Pereira et al., 2001; Molk et al., 2004).

In this paper, we investigate the role of Bub2/Bfa1 localization at SPBs in controlling mitotic exit. Our data indicate that Bub2/Bfa1 disappearance from the mother-bound SPB at the onset of anaphase, along with Cdc5 function, helps prompt Tem1 activation in telophase. This asymmetric disappearance of Bub2/Bfa1 requires Bub2 GAP activity and depends on a functional septin ring, consistent with the notion that passage of the daughter-oriented SPB through the bud neck signals mitotic exit (Molk et al., 2004). Altogether, our data highlight a new molecular mechanism coupling MEN activation with passage of the nucleus through the bud neck.

Results

Symmetrically localized Bub2-myc9 is lethal to some MEN-defective mutants

Because activation of the spindle position checkpoint leads to the persistence of Bub2/Bfa1 on both SPBs (Pereira et al., 2001; Molk et al., 2004), symmetric distribution of the GAP could contribute to keeping Tem1 inactive. In fact, Tem1 is also present on both SPBs during unperturbed anaphase (Molk et al., 2004). We therefore asked whether Bub2's disappearance from the SPB remaining in the mother cell could be important for proper mitotic exit.

We previously showed that a modified version of Bub2 with nine myc epitopes at the COOH terminus (Bub2-myc9) localizes symmetrically on both SPBs throughout the cell cycle (Fraschini et al., 1999), unlike the GFP-tagged counterpart (Pereira et al., 2000). This difference was attributed to the assay we used at that time (Pereira et al., 2000), which relied on a chromosome-spreading technique. We therefore reinvestigated Bub2 subcellular localization by indirect immunofluorescence on fixed cells of strains expressing either Bub2-myc9 or the Bub2-HA3 and Bub2-HA6 variants, carrying three and six HA epitopes, respectively, at the COOH terminus. We found that Bub2-HA3 (Fig. 1 A) localized asymmetrically on the SPB moving into the bud in 93% (±1.3%, $n = 289$) of the cells undergoing anaphase, similar to Bub2-HA6 (not depicted), whereas Bub2-myc9 was present on both SPBs in 88.3% (±7.9, $n = 408$) of the cells in the same stage of the cell cycle (Fig. 1 B). Therefore, symmetric localization is a peculiarity of Bub2-myc9,

Table I. **Genetic interactions between *BUB2* alleles and mutations affecting the MEN**

Strain	*bub2Δ*	*BUB2-HA3*	*BUB2-myc9*
cin8Δ	−	+	+
cdc5-1	+	+	−
cdc5-2	+	+	−
cdc5-3	+	+	−
tem1-3	+	+	−
nud1-44	+	+	−
cdc14-3	+	ND	+
cdc15-2	+	ND	+
dbf2-2	+	ND	+
lte1Δ	+	ND	+
cla4Δ	+	ND	+/−
BFA1-11A	ND	ND	+

Strains carrying the *BUB2* alleles indicated in the first row were crossed with a *cin8Δ* strain as a control of checkpoint proficiency and with the MEN-defective mutants listed in the left column, followed by sporulation and tetrad dissection on YEPD. At least 12 tetrads were analysed for each cross. + indicates the ability to form normal-sized colonies, − indicates no growth, and +/− indicates a synthetic growth effect of segregants carrying the indicated allele combinations after 3 d at 25°C. In all cases, the frequency of double mutants was that expected from the segregation of two unlinked markers (25%), and the indicated phenotypes were 100% penetrant.

rather than an artifact attributable to the immunostaining procedure. Because Bub2 forms a complex with Bfa1 and either protein is necessary for proper localization of the other at SPBs (Pereira et al., 2000), we analyzed the localization of a fully functional Bfa1 variant tagged with six HA epitopes (Bfa1-HA6) in cells expressing Bub2-myc9 as the only Bub2 source. As previously shown (Pereira et al., 2000), Bfa1-HA6 was asymmetrically localized on the bud-directed SPB in 91.8% (±4.1%, $n = 319$) of wild-type anaphase cells (Fig. 1 A), whereas it was found on both SPBs in 58.2% (±10.6%, $n = 446$) of *BUB2-myc9* anaphase cells (Fig. 1 B), indicating that Bub2-myc9's persistence on the mother cell SPB prevents Bfa1's disappearance from the same SPB in many anaphase cells (Fig. 1 B). Similarly, a Tem1-HA3–tagged protein was found symmetrically localized on both SPBs in 83.8% (±0.8%, $n = 251$) of anaphase cells expressing Bub2-myc9 (Fig. 1 B), whereas it was present on both SPBs in only 27.2% (±1.0%, $n = 174$) of wild-type anaphase cells (Fig. 1 A).

Symmetric localization of Bub2/Bfa1 did not cause any obvious cell cycle defect in otherwise wild-type cells (unpublished data). However, because Tem1 activation is likely regulated by redundant mechanisms, we looked for synthetic effects between Bub2-myc9 and mutations affecting the MEN. As shown in Table I, although Bub2-myc9 was perfectly tolerated by mutants defective in MEN proteins acting downstream of Tem1, such as *cdc15-2*, *dbf2-2*, and *cdc14-3*, it was lethal at the permissive temperature for *tem1-3*, *nud1-44*, *cdc5-1*, *cdc5-2*, and *cdc5-3* mutants, suggesting that its presence is toxic when Tem1 activation is impaired. In fact, Nud1 is thought to act as an anchor for Tem1 and other MEN components at SPBs, whereas Cdc5 promotes Tem1 activation at different levels (Simanis, 2003; Stegmeier and Amon, 2004). Surprisingly, *BUB2-myc9* was not toxic for *lte1Δ* cells, presumably because Lte1 is dispensable for mitotic exit at 25°C (Shirayama et al., 1994; Bardin et al., 2000; Pereira et al., 2000; Hofken and Schiebel, 2002; Seshan et al., 2002; Yoshida et al., 2003), whereas it caused a synthetic growth defect to *cla4Δ* cells that are defective in mitotic exit and in Lte1 activation (Hofken and Schiebel, 2002; Seshan et al., 2002; Chiroli et al., 2003). Noticeably, deletion of *BUB2* was not lethal for any of the aforementioned mutants, indicating that the synthetic lethality of *BUB2-myc9* with the *tem1*, *cdc5*, and *nud1* conditional alleles is not attributable to *BUB2*'s loss of function. Accordingly, unlike *BUB2* deletion, expression of *BUB2-myc9* as the only Bub2 source in the cells did not impair checkpoint activation (Fraschini et al., 1999) and did not cause lethality to cells lacking the kinesin Cin8 (Table I). Moreover, the *BUB2-HA3* allele, whose product localizes asymmetrically on SPBs during anaphase, was perfectly tolerated by *tem1*, *nud1*, and *cdc5* mutants (Table I). Thus, Bub2-myc9 behaves like a gain-of-function variant downregulating Tem1, and its deleterious effects might be linked to symmetric SPB localization of Bub2/Bfa1.

BUB2-myc9 is a dominant allele causing Bfa1-dependent mitotic exit defects in *cdc5-2* cells

To uncover the defects caused by Bub2-myc9 in MEN mutants, we chose to make a conditionally lethal *cdc5-2 BUB2-myc9* strain. Because Cdc5 is an unstable protein, we generated a *cdc5-2 BUB2-myc9* strain carrying a wild-type copy of *CDC5* under the control of the Met-repressible *MET3* promoter. As shown in Fig. 1 C, *MET3-CDC5 cdc5-2 BUB2-myc9* cells were viable in medium lacking Met, where *MET3-CDC5* is expressed, but they were unviable in Met-containing medium at 25°C (permissive temperature for *cdc5-2*). This behavior did not change when one extra copy of *BUB2* was integrated into the genome of *BUB2-myc9 cdc5-2 MET3-CDC5* cells (Fig. 1 C), indicating that *BUB2-myc9* is a dominant allele.

To analyze the terminal phenotype caused by the *BUB2-myc9 cdc5-2* combination, cell cultures of wild-type, *cdc5-2 MET3-CDC5*, and *BUB2-myc9 cdc5-2 MET3-CDC5* strains, exponentially growing in the absence of Met, were synchronized in G1 with α factor and released at 25°C into fresh medium containing Met to shut off *CDC5* expression. The pheromone was added back 120 min after release to prevent cells from entering a second cell cycle. FACS profiles of DNA contents (Fig. 2 A) and analysis of nuclear division and spindle elongation (Fig. 2 B) showed that *cdc5-2* cells progressed normally through the cell cycle with kinetics similar to wild-type cells. Conversely, *BUB2-myc9 cdc5-2* cells accumulated with 2C DNA content, two divided nuclei, and long anaphase spindles. In addition, unlike *cdc5-2* cells, they failed to bring about Clb2 proteolysis, Sic1 reaccumulation, and inactivation of the Clb2/Cdk1 kinase (Fig. 2 C), indicating that mitotic exit was compromised. In some experiments, spindles could eventually disassemble at 210–240 min after release from the G1 arrest, although cells could neither undergo cytokinesis nor rebud and re-replicate. Overexpression of *BUB2* from the galactose-inducible *GAL1* promoter had similar effects in *cdc5-2* cells as the presence of Bub2-myc9. In fact, *cdc5-2* cells expressing *GAL1-BUB2* were unviable in galactose-containing medium

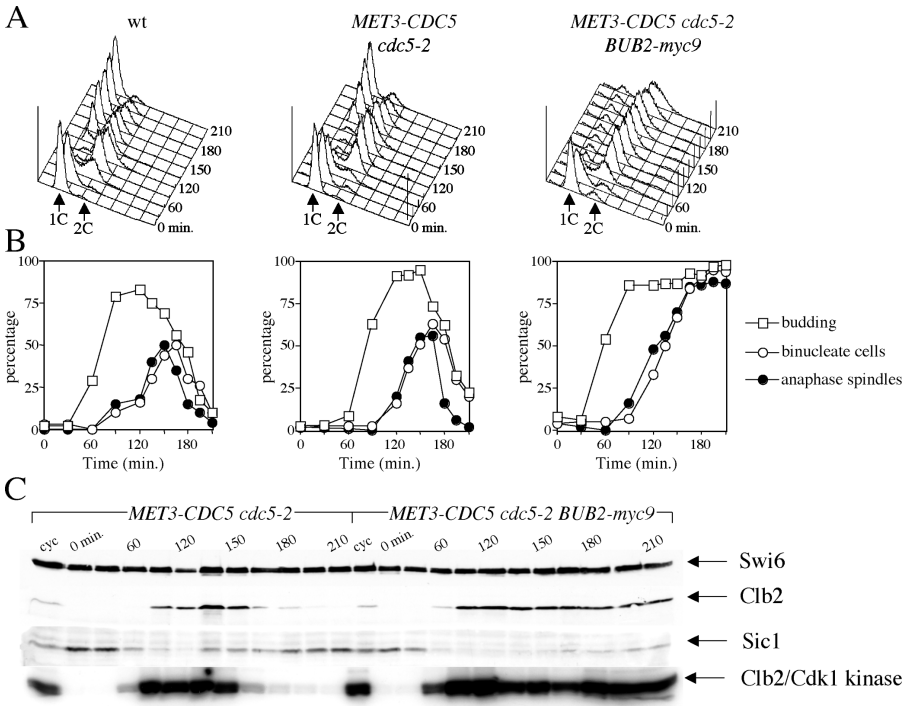


Figure 2. **Expression of Bub2-myc9 delays mitotic exit in *cdc5-2* cells at permissive temperature.** Cultures of wild-type (ySP41), *MET3-CDC5 cdc5-2* (ySP3426), and *MET3-CDC5 cdc5-2 BUB2-myc9* cells (ySP3480), exponentially growing in –Met medium, were arrested in G1 with α factor and released at 25°C in YEPD supplemented with 2 mM Met (*MET3-CDC5* expression off). After 120 min, 10 μg/ml α factor was readded to prevent cells from entering a second cell cycle. Cells were collected at the indicated times for FACS analysis of DNA contents (A); kinetics of budding, nuclear division, and spindle elongation/breakdown after in situ immunostaining of tubulin (B); and Western analysis of Clb2 and Sic1, as well as Clb2/Cdk1 immunoprecipitations followed by kinase assays using histone H1 as substrate (C). Swi6 was used as loading control.

at the permissive temperature and accumulated in telophase with 2C DNA content, two divided nuclei, and long anaphase spindles (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200507162/DC1), whereas overexpression of the same construct in wild-type cells caused no growth defect and at most a 15-min delay in mitotic exit compared with the isogenic untransformed strain. Thus, high levels of wild-type Bub2 recapitulate the effects of Bub2-myc9 in *cdc5-2* cells, consistent with the notion that *BUB2-myc9* is a gain-of-function allele.

Because Bub2 acts in a complex with Bfa1, which is required for Bub2 localization at SPBs (Pereira et al., 2000), deletion of *BFA1* could be expected to bypass the mitotic exit delay of *BUB2-myc9 cdc5-2* cells. Indeed, *MET3-CDC5 cdc5-2 BUB2-myc9 bfa1Δ* cells released from a G1 block in the presence of Met could exit mitosis, disassemble spindles, and reaccumulate mononucleate cells with kinetics similar to those of *MET3-CDC5 cdc5-2* cells under the same conditions (Fig. 3), indicating that Bfa1 is required for Bub2-myc9 to exert its inhibitory function on mitotic exit in *cdc5-2* cells.

Whereas the MEN is absolutely required for Cdc14 activation, mutants defective in the FEAR pathway only delay mitotic exit (for review see Stegmeier and Amon, 2004). We therefore asked whether Bub2-myc9 could further compromise mitotic exit in separase *esp1-1* mutant cells at the nonpermissive temperature. Separase inactivation at 37°C prevents sister chromatid separation (Ciosk et al., 1998) and mildly delays mitotic exit, allowing the undivided nuclei to enter a new round of DNA replication and accumulate with DNA contents higher than 2C (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). Expression of Bub2-HA6, which was asymmetrically localized on SPBs, did not affect cell cycle progression of *esp1-1* cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200507162/DC1). Conversely, expression of Bub2-myc9, which was present on both SPBs in most *esp1-1* cells, prevented them from undergoing mitotic exit and entry into a new round of DNA replication (Fig. S2). Thus, the constitutive presence of Bub2/Bfa1 at both SPBs might inhibit mitotic exit when the FEAR pathway is compromised by separase inactivation. In addition, because the mitotic exit of *esp1-1* cells has been attributed to the peculiar migration of the undivided nuclei and embedded SPBs into the bud (McGrew et al., 1992), which would lead to Tem1 exposure to Lte1 (Bardin et al., 2000), our data argue that the encounter between Lte1 and Tem1 in the bud is not sufficient to promote mitotic exit when Bub2-myc9 is present in the cells, and therefore the Bub2–Bfa1 complex is symmetrically localized on SPBs.

MEN hyperactivation can rescue the inhibitory effects of Bub2-myc9

Because expression of Bub2-myc9 prevented mitotic exit when either the MEN or the FEAR pathway was partially impaired, we directly tested to determine whether MEN hyperactivation by different means could bypass the mitotic exit defect of *BUB2-myc9 cdc5-2* cells. As shown in Fig. 4 A, high levels of the Tem1 activator Lte1 can counteract the deleterious effects of Bub2-myc9 because galactose induction of a *GAL1-LTE1* fusion could suppress the *BUB2-myc9 cdc5-2* synthetic lethality, tipping the balance in favor of Tem1 activation. In addition, the *CDC14^{TAB6-1}* allele (Shou et al., 2001), encoding a constitutively active variant of the downstream MEN target, restored viability of *BUB2-myc9 cdc5-2* cells (Fig. 4 B), consistent with their failure to activate the MEN. Conversely, deletion of either *AMN1* or *DMA1* and *DMA2*, whose gene products counteract MEN activation (Wang et al., 2003; Fraschini et al., 2004), did not suppress *BUB2-myc9 cdc5-2* (unpublished data). Remarkably,

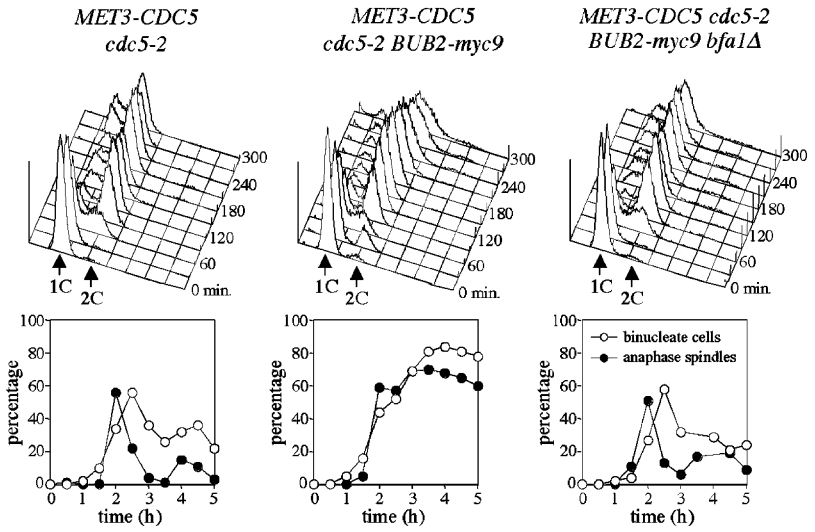


Figure 3. **The mitotic exit delay of *cdc5-2 BUB2-myc9* cells depends on functional Bfa1.** Strains with the indicated genotypes (ySP3426, ySP3480, and ySP3481) were grown in –Met medium, arrested in G1 with α factor, and released at 25°C in the presence of Met. Cells were collected at the indicated time points for FACS analysis of DNA contents (top) and to measure kinetics of nuclear division and spindle elongation/breakdown (bottom) as described in Fig. 2 B.

TEM1 suppressed the lethal effects of the *BUB2-myc9 cdc5-2* combination not only when expressed from either the *GAL1* promoter or from its attenuated version *GALs* (Fig. 4 A) but even when just one extra copy was expressed from its own promoter, suggesting that Tem1 becomes limiting in *cdc5-2 BUB2-myc9* strains (Fig. 4 A). It is important to notice that overexpressed Tem1 did not disrupt the symmetric localization of Bub2-myc9 (unpublished data), suggesting that suppression is not attributable to Bub2 titration from SPBs.

Bub2 GAP activity is not required to inhibit Tem1 but promotes Bub2/Bfa1 disappearance from the mother-bound SPB

We investigated whether the toxic effects of Bub2-myc9 on mitotic exit were attributable to increased GAP activity on Tem1 by using bacterially expressed and purified 6×His-Tem1, maltose binding protein (MBP)–Bfa1, and GST-Bub2 fusions in a previously described in vitro GAP assay (Geymonat et al., 2002). The rate of GTP hydrolysis and dissociation together was measured using Tem1 bound to γ-[^{32}P]GTP, whereas the rate of GTP dissociation alone was measured using Tem1 bound to the nonhydrolyzable GTP analogue γ-[^{35}S]GTP. As reported previously (Geymonat et al., 2002), Tem1 showed on its own GTPase activity and, to a lower extent, GTP release (compare Fig. 5 A with Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200507162/DC1). The presence of Bfa1 stabilized Tem1 in the GTP-bound form, whereas Bub2 had little or no effect on its own (Fig. 5 A and Fig. S3). As previously shown (Geymonat et al., 2002), Bub2 stimulated Tem1 GTPase activity in the presence of Bfa1 (Fig. 5 A) but not GTP dissociation (Fig. S3). We then compared the GAP activity of purified GST-Bub2, GST-Bub2-HA3, and GST-Bub2-myc9. Surprisingly, Bub2-myc9 could not stimulate the GAP activity of GTP-bound Tem1, whereas Bub2-HA3 was as active as untagged Bub2 (Fig. 5 B). Assaying the GTPase activity of Tem1 in the presence of Bfa1 and increasing amounts of Bub2 or Bub2-myc9 confirmed that Bub2-myc9 is unable to stimulate Tem1 GTPase activity at any tested concentration (Fig. 5 C). These findings, along with the observation that Bub2-myc9 is proficient in activating the spindle positioning checkpoint (Fraschini et al., 1999), argue that Bub2 GAP activity is dispensable for MEN inhibition.

However, the GAP activity of Bub2 might be required to control SPB localization of the Bub2–Bfa1 complex. To investigate this possibility, we generated a mutant Bub2 variant, Bub2R85A, where an alanine residue replaces arginine 85, which appears to be the catalytic arginine in the GAP domain according to sequence comparison with other GAPs (Neuwald, 1997; Albert et al., 1999). As shown in Fig. 5 (C and D), the R85A substitution completely abolished the in vitro GAP activity of both untagged and HA-tagged Bub2, whereas it could not further affect the already impaired activity of Bub2-myc9.

We then replaced the endogenous *BUB2* gene with the *bub2R85A-HA3* allele in a haploid yeast strain and analyzed the localization of the corresponding protein by in situ immunofluorescence. Unlike Bub2-HA3 and similar to Bub2-myc9, Bub2R85A-HA3 remained on both SPBs after the onset of anaphase in 75% of the cells (Fig. 5 E), indicating that Bub2 GAP activity is required to promote Bub2 release from the mother-bound SPB at the metaphase–anaphase transition. Remarkably, the R85A substitution completely knocked out the checkpoint function of Bub2, Bub2-HA3, and Bub2-myc9. In fact, like *bub2Δ* cells, *bub2R85A* cells were hypersensitive to the microtubule depolymerizing compound benomyl (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200507162/DC1) and re-replicated their DNA in the presence of nocodazole (Fig. S4 C), indicating that checkpoint response to spindle disruption is completely abolished. In addition, like *bub2Δ* cells, they were unable to activate the spindle position checkpoint, as judged by their ability to rebud in the presence of misoriented spindles (Fig. S4 B), caused by deletion of *DYN1* (Yeh et al., 1995) and *KAR9* (Korinek et al., 2000; Lee et al., 2000). Therefore, because both the addition of the myc epitopes and the R85A substitution impair Bub2 GAP activity, the checkpoint defects observed in the R85A mutants should involve some Bub2 features other than GAP. Interestingly, the R85A substitution did not affect either the interaction of Bub2-myc9 and -HA3 with Tem1-GFP (Fig. S4 D) or the ability of Bub2-myc9 to pull down Bfa1-HA6 and vice versa (Fig. 5 G). However, unlike Bub2-myc9, Bub2R85A-myc9

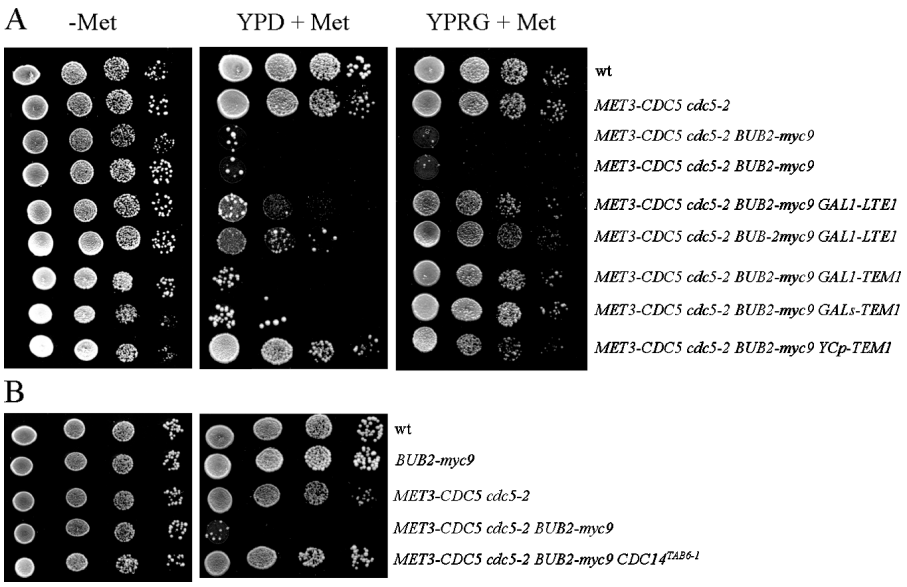


Figure 4. **Hyperactivation of the MEN restores viability of *BUB2-myc9 cdc5-2* cells.** Serial dilutions of wild-type (ySP41), *MET3-CDC5 cdc5-2* (ySP3426), *MET3-CDC5 cdc5-2 BUB2-myc9* (ySP3442 and ySP3480), *MET3-CDC5 cdc5-2 BUB2-myc9 GAL1-LTE1* (ySP3482 and ySP3495), *MET3-CDC5 cdc5-2 BUB2-myc9 GAL1-TEM1* (ySP3646), *MET3-CDC5 cdc5-2 BUB2-myc9 GALs-TEM1* (ySP3904), *MET3-CDC5 cdc5-2 BUB2-myc9* carrying a *TEM1*-bearing centromeric plasmid (YCp-*TEM1*), and *MET3-CDC5 cdc5-2 BUB2-myc9 CDC14*$^{TAB6-1}$ (ySP3891) strains, grown in –Met medium (*MET3-CDC5* on), were spotted on –Met, YEPD +Met (*MET3* and *GAL1/GALs* promoters off), and YEPRG +Met (*MET3-CDC5* off, *GAL1/GALs* promoter on) plates, which were incubated at 25°C for 2 d.

failed to recruit Bfa1 at SPBs at any cell cycle stage (Fig. 5 F), in spite of its presence on both SPBs throughout the cell cycle (unpublished data), thus providing an explanation for its inability to engage the checkpoint. Altogether, our data suggest that Bub2 GAP activity is not directly involved in Tem1 inactivation but is rather required to regulate Bub2 and Bfa1 asymmetrical localization at anaphase.

Factors that regulate the disappearance of Bub2 from the mother-bound SPB

To gain insights into the mechanisms that regulate Bub2/Bfa1 disappearance from the mother-bound SPB at the onset of anaphase and their connections with mitotic exit control, we analyzed the distribution of Bub2-HA3 in different mutants. Because both interaction of cytoplasmic microtubules with the bud cortex (Adames et al., 2001; Pereira et al., 2001) and passage of the daughter-oriented SPB through the bud neck (Molk et al., 2004) have been proposed to signal mitotic exit, we selected mutants on the basis of their possible defects in the following processes: bud neck formation/localization, SPB regulation/localization, microtubule dynamics, and MEN activation. Because some of the analyzed mutations affect spindle positioning and therefore cause Bub2-HA3 to be maintained on both SPBs, the percentage of asymmetric versus symmetric Bub2-HA3 localization was scored only in cells undergoing properly oriented anaphase, as determined by DAPI staining of nuclei. The asymmetric localization of Bub2-HA3 was not disrupted by mutations in genes encoding the following proteins (unpublished data): β-tubulin (*tub2-104*); the kinesins Cin8 and Kip3 (*cin8Δ* and *kip3Δ*); the SPB components Cnm67 and Spc72 (*cnm67Δ* and *spc72-7*); the bud cortical proteins Kar9, Gic1, and Gic2 (*kar9Δ* and *gic1Δ gic2Δ*); the MEN components Cdc5 and -14; and the B-type cyclins Clb3 and -4, which have been shown to be localized asymmetrically on SPBs (Liakopoulos et al., 2003). Conversely, lack of the plus-end microtubule binding protein Bim1 increased the fraction of anaphase cells with Bub2-HA3 on both SPBs from 10 to 25% (Fig. 6 A), suggesting that Bim1 contributes to the signal disappearance of Bub2 from the mother-bound SPB in anaphase. Strikingly, we found that protein kinases localized at the bud neck, namely Swe1, Gin4, and Hsl1, participate in promoting Bub2-HA3 disappearance from the mother-bound SPB. In fact, the fraction of anaphase cells with symmetrically localized Bub2-HA3 increased from 10 to 43, 18, and 28% in *swe1Δ*, *gin4Δ*, and *hsl1Δ* mutants, respectively (Fig. 6 A). Deletion of *SWE1* further increased the percentage of *gin4Δ* and *hsl1Δ* cells with Bub2-HA3 on both SPBs (Fig. 6 A), pointing to Swe1 as an important determinant for Bub2 asymmetry during anaphase. Consistent with a role for the bud neck in regulating Bub2/Bfa1 localization, Swe1-lacking cells where the septin ring was disrupted by either the *cdc12-1* septin mutation or overexpression of the dominant-negative *CLA4t* allele (Chiroli et al., 2003) led to symmetric localization of Bub2-HA3 at SPBs in 64 and 70% of anaphase cells, respectively. This result can be partly explained by the failure of these mutants to properly activate or localize at the bud neck the kinases Gin4, Hsl1, and Swe1, whose recruitment to the bud neck depends on septins (Carroll et al., 1998; Barral et al., 1999; Longtine et al., 2000). Therefore, bud neck components are necessary to signal Bub2 elimination from the mother cell SPB when the spindle is properly oriented. To assess the effects of Bub2 retention at both SPBs on mitotic exit, we analyzed kinetics of spindle disassembly in *swe1Δ* cells overexpressing *CLA4t*. As shown in Fig. 6 B, spindle disassembly, and therefore mitotic exit, was markedly delayed in these cells compared with wild type. It should be noted that, by 5 h, spindles had been correctly oriented in virtually all anaphase cells, suggesting that the lack of spindle disassembly in ∼50% of the cells was not attributable to spindle misorientation but rather to the persistence of Bub2 on both SPBs because it was abolished by *BUB2* deletion.

Similar to what we found with Bub2-myc9, both expression of *CLA4t* and the *cdc12-1* mutation caused growth defects in *cdc5-2* cells at the permissive temperature, and these synthetic effects could be rescued by deleting *BUB2* or *BFA1*

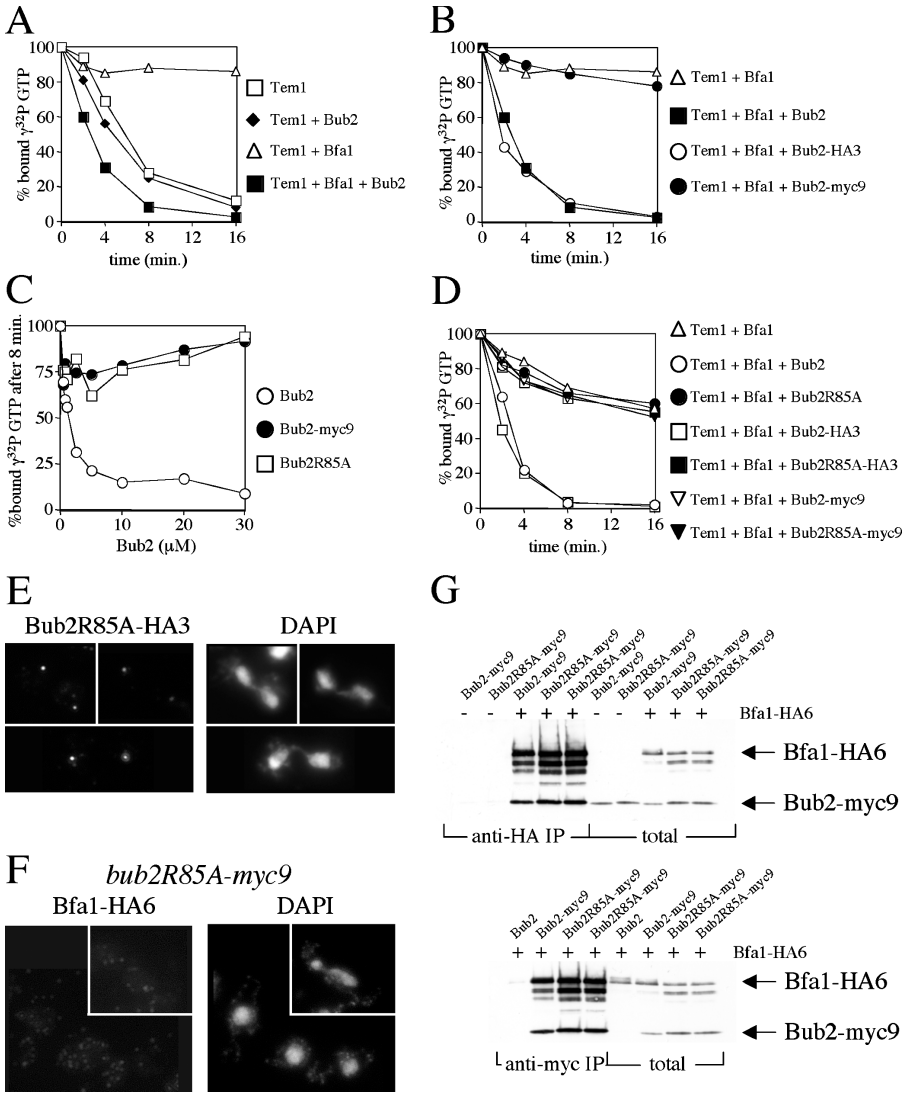


Figure 5. **Lack of Bub2 GAP activity leads to persistent symmetric localization of Bub2 on SPBs.** (A, B, and D) Bacterially expressed GST-Bub2, MBP-Bfa1, and 6×His-Tem1 were used in in vitro GAP assays as previously described (Geymonat et al., 2002). In brief, 240 nM of 6×His-Tem1 was loaded with γ-[^{32}P]GTP in either the absence or presence of 150 nM MBP-Bfa1 and incubated at 30°C for 10 min. The mixture was then added to 15 μM of GST-Bub2 or buffer alone, and kinetics of GTP hydrolysis and dissociation was followed by filter binding assays (see Materials and methods). (C) Increasing amounts of the indicated Bub2 variants (0:1, 1:1, 2:1, 2.6:1, 6.6:1, 13:1, 26:1, 53:1, and 80:1 molar ratio Bub2/Bfa1) were added to 605 nM of 6×His-Tem1–γ-[^{32}P]GTP in the presence of 378 nM MBP-Bfa1 (final concentrations), and the fraction of filter-bound radioactivity was measured after 8 min. (E) Localization of Bub2R85A-HA3 at anaphase SPBs was monitored by in situ immunostaining with anti-HA antibodies. (F) Localization of Bfa1-HA6 in asynchronous *BUB2R85A-myc9* cells was monitored as in E. (G, top) Protein extracts from *BUB2-myc9* (ySP710), *bub2R85A-myc9* (ySP4696), *BUB2-myc9 BFA1-HA6* (ySP5087), and two different *bub2R85A-myc9 BFA1-HA6* strains (ySP5118 and ySP5119) were used for Western blot analysis of either the total levels of Bub2-myc9 and Bfa1-HA6 (total) or the amount of Bub2-myc9 coimmunoprecipitating with Bfa1-HA6 (anti-HA IP). (bottom) Protein extracts from *BFA1-HA6* (ySP2035), *BUB2-myc9 BFA1-HA6* (ySP5087), and two different *bub2R85A-myc9 BFA1-HA6* strains (ySP5118 and ySP5119) were used for Western blot analysis of either the total levels of Bub2-myc9 and Bfa1-HA6 (total) or the amount of Bfa1-HA6 coimmunoprecipitating with Bub2-myc9 (anti-myc IP).

(Fig. 6, C and D), further supporting the notion that symmetrically localized Bub2 is deleterious for mitotic exit when Cdc5 is not fully functional.

Discussion

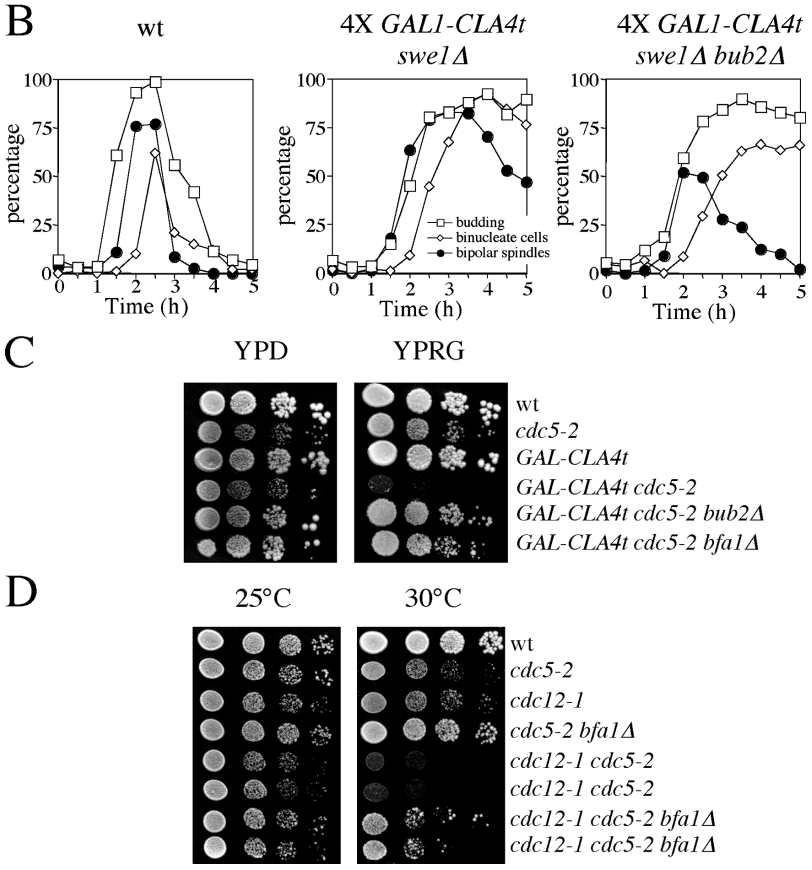
Disappearance of Bub2/Bfa1 from the mother-bound SPB as a trigger for mitotic exit

Eukaryotic cells that divide asymmetrically must prevent mitotic exit and cytokinesis when the spindle is misoriented with respect to the cell division axis. This represents a major issue for budding yeast, where assembly of the bud neck at the G1–S transition defines the site of cell division, compared with other organisms that establish the cleavage plane only after bipolar spindle formation. Therefore, it is not surprising that the GTPase Tem1 is finely regulated, as it triggers mitotic exit and cytokinesis in budding yeast through MEN activation. Tem1 is kept inactive throughout most of the cell cycle by the Bub2–Bfa1 complex, which normally localizes with Tem1 at the bud-directed spindle pole from the anaphase onset to the end of mitosis. Regulation of the MEN on the mother-bound

Figure 6. **Disappearance of Bub2 from the mother-bound SPB depends on proteins localized at the bud neck.** (A) Localization of Bub2-HA3 was analyzed on formaldehyde-fixed cells undergoing properly oriented anaphase (as assessed by DAPI staining) from logarithmically growing cultures at 25°C, with the exception of *BUB2-HA3* 4× *GAL1-CLA4t swe1Δ* cells, which were grown in raffinose-containing medium, arrested in G1 by α factor, and released in the presence of galactose for 3 h to induce *GAL1-CLA4t* expression. At least 150 anaphase cells were scored for each strain. (B) Wild-type (ySP41), 4× *GAL1-CLA4t swe1Δ* (ySP2711), and 4× *GAL1-CLA4t swe1Δ bub2Δ* (ySP2728) cells were grown in YEPR at 25°C, arrested in G1 with α factor, and released in YEPRG at 25°C. Galactose was added 30 min before the release. 120 min after the release, 10 μg/ml α factor was readded to prevent cells from entering a second cell cycle. Cells were collected at the indicated times for kinetics of budding, nuclear division, and bipolar spindle formation/breakdown after in situ immunostaining of tubulin. Bipolar spindles include both metaphase and anaphase spindles. (C) Serial dilutions of wild-type (ySP41), *cdc5-2* (ySP324), *GAL1-CLA4t* (ySP2622), *GAL1-CLA4t cdc5-2* (ySP3565), *GAL1-CLA4t cdc5-2 bub2Δ* (ySP4802), and *GAL1-CLA4t cdc5-2 bfa1Δ* (ySP4804) cell cultures were spotted on either YPD (*GAL1* promoter off) or YPRG (*GAL1* promoter on) plates and incubated at 25°C for 3 d. (D) Serial dilutions of wild type (ySP41), *cdc5-2* (ySP324), *cdc12-1* (ySP293), *cdc5-2 bfa1Δ* (ySP3671), *cdc12-1 cdc5-2* (ySP4473 and ySP4474), and *cdc12-1 cdc5-2 bfa1Δ* (ySP4518 and ySP4519) were spotted on YPD plates, which were incubated at 25 and 30°C for 2 d.

A

Relevant genotype	asymmetric localization (%)	symmetric localization (%)
BUB2-HA3	90	10
BUB2-HA3 bim1Δ	75	25
BUB2-HA3 cdc12-1	80	20
BUB2-HA3 cdc12-1 swe1Δ	36	64
BUB2-HA3 swe1Δ	57	43
BUB2-HA3 4X *GAL1-CLA4t swe1Δ*	30	70
BUB2-HA3 gin4Δ	82	18
BUB2-HA3 gin4Δ swe1Δ	66	34
BUB2-HA3 hsl1Δ	72	28
BUB2-HA3 hsl1Δ swe1Δ	69	31



SPB might have an important role in controlling mitotic exit because activation of the spindle position checkpoint, which prevents mitotic exit, preserves symmetric Bub2/Bfa1 localization at SPBs (Pereira et al., 2001; Molk et al., 2004). On the other hand, unlike Bub2/Bfa1, Tem1 is also found on the mother-bound SPB when anaphase takes place properly (Molk et al., 2004).

Our characterization of the gain-of-function *BUB2-myc9* allele that allows localization of Bub2/Bfa1 on both SPBs throughout the cell cycle provides new evidence that Bub2/Bfa1 removal from the spindle pole staying in the mother cell contributes to triggering mitotic exit. The role of Bub2/Bfa1 disappearance from the mother-bound SPB in mitotic exit seems to overlap with other ways of activating Tem1, as it becomes apparent only when Tem1 itself or the scaffold spindle pole component Nud1 or the polo kinase Cdc5 function are crippled. In addition, we found that Bub2-myc9 is also lethal to cells overexpressing *DMA2* (unpublished data), which we recently revealed to be involved in Tem1 inactivation (Fraschini et al., 2004).

Nud1 activates the MEN by recruiting Tem1 and other MEN components to SPBs (Gruneberg et al., 2000), whereas Cdc5 triggers mitotic exit through different mechanisms (for review see Stegmeier and Amon, 2004). On the other hand, the observation that temperature sensitivity of the *cdc5-1* and *-2* mutants used in this study can be partially rescued by deletion of *BFA1* (Hu et al., 2001) suggests that they are impaired in Tem1 activation. Consistently, higher *TEM1* dosage suppresses *cdc5-2 BUB2-myc9* lethality, indicating that the latter is likely attributable to either Tem1 sequestration or a failure to properly activate it.

Although direct inhibitory phosphorylation seems to be a major function of Cdc5 in promoting mitotic exit, a mutant

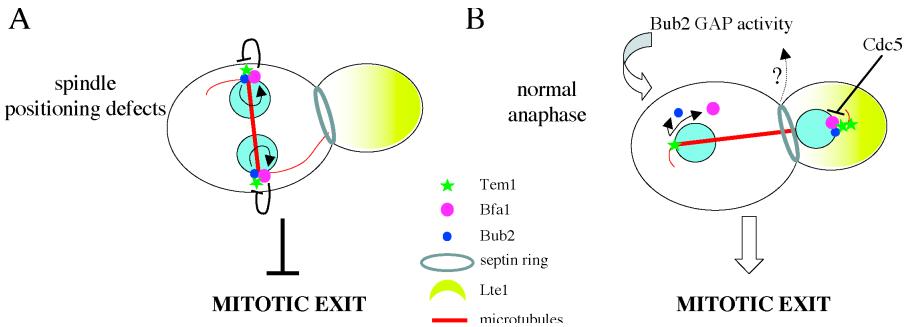


Figure 7. **A schematic model for mitotic exit.** In the presence of spindle positioning defects (A), Bub2-dependent Bfa1 retention at both SPBs prevents mitotic exit by inhibiting Tem1. When anaphase takes place correctly and one SPB crosses the bud neck (B), a signal is sent to the mother-bound SPB, where the Bub2–Bfa1 complex is removed through the action of Bub2 GAP activity, thus leading to Tem1 activation at this pole. This helps trigger mitotic exit, together with Bfa1 inhibition by Cdc5, presumably at the bud-directed SPB, and with increased Tem1 loading on the same SPB.

version of Bfa1 no longer phosphorylatable by Cdc5 (Bfa1-11A; Hu et al., 2001) was not synthetically lethal with Bub2-myc9 (Table I), indicating that Cdc5 likely regulates Bfa1 function by additional means besides direct phosphorylation. Another task that Cdc5 might carry out to promote mitotic exit is phosphorylation of the Tem1 activator Lte1 (Lee et al., 2001). However, *LTE1* deletion does not cause synthetic effects when combined with the *BUB2-myc9* allele (Table I). Therefore, Cdc5 might contribute to MEN activation and/or Bub2/Bfa1 inhibition through redundant mechanisms. One of them is likely related to the involvement of Cdc5 in the FEAR pathway (Stegmeier et al., 2002; Yoshida et al., 2002). Accordingly, when we inactivated the latter by the *esp1-1* separase mutant allele, Bub2-myc9 prevented mitotic exit (Fig. S2).

Coupling between mitotic exit and nuclear partitioning has been proposed to be triggered by exposure of Tem1, carried by the daughter-oriented SPB, to Lte1, which is constrained in the bud (Bardin et al., 2000; Bloecher et al., 2000; Pereira et al., 2000). Although this mechanism might be important for Tem1 activation in late anaphase at low temperatures, our data suggest that it might not be sufficient to drive mitotic exit when Bub2/Bfa1 remains symmetrically localized on SPBs. In fact, mitotic exit and entry into a new round of DNA replication do not take place in *esp1-1* mutant cells when Bub2/Bfa1 is present on both SPBs, in spite of the encounter between Tem1 and Lte1. Accordingly, Lte1 was found to be dispensable for the unscheduled mitotic exit of mutants defective in the spindle position checkpoint (Adames et al., 2001). We therefore propose that disappearance of Bub2/Bfa1 from the mother-bound SPB contributes to couple mitotic exit with properly oriented chromosome partitioning. Interestingly, a role for the mother cell in controlling the MEN has recently been highlighted by the finding that the Kin4 kinase, involved in the spindle position checkpoint, is specifically localized in the mother cell (D'Aquino et al., 2005; Pereira and Schiebel, 2005).

A novel view for Bub2's role in controlling mitotic exit

The Bub2–Bfa1 complex is proposed to prevent mitotic exit by stimulating Tem1 GTPase activity both in budding and in fission yeast (Simanis, 2003; for review see Stegmeier and Amon, 2004). According to this hypothesis, knocking down the GAP activity of the complex should allow Tem1 activation even in conditions triggering a checkpoint response, i.e., microtubule defects or spindle misorientation. Because Bub2 but not Bfa1 carries a conserved GAP domain, we directly tested this hypothesis by substituting the putative catalytic arginine (R85) with alanine (Neuwald, 1997; Albert et al., 1999). Indeed, unlike wild-type Bub2, Bub2R85A completely lacked in vitro GAP activity and caused checkpoint defects similar to *BUB2* deletion. Surprisingly, we also found that Bub2-myc9 had no detectable in vitro GAP activity, although it could normally support the checkpoint, suggesting that Bub2 likely contributes to Tem1 inhibition by means other than stimulating its GTPase activity. It is important to emphasize that we and others (Geymonat et al., 2002) have shown that Tem1 on its own has a high rate of GTP hydrolysis, as well as guanosine 5′-diphosphate (GDP) release, unlike other Ras-like G proteins. In agreement with Tem1's ability to switch by itself between GTP- and GDP-bound forms, Lte1 mitotic exit function does not seem to be related to its putative guanine nucleotide exchange factor activity on Tem1 (Yoshida et al., 2003). Rather, it could be linked to its ability to stimulate Tem1 recruitment to the daughter-directed SPB after anaphase (Molk et al., 2004).

Thus, among several possible models, we favor the idea that Bfa1 alone is responsible for Tem1 inhibition in *BUB2-myc9* cells (Fig. 7 A). Indeed, Bfa1 has been shown to be able to inhibit Tem1 and mitotic exit independently of Bub2 (Li, 1999; Ro et al., 2002), perhaps by preventing its cycling between GTP and GDP binding (Geymonat et al., 2002) and/or by inhibiting its binding to Cdc15 (Ro et al., 2002). If Bfa1 alone can account for Tem1 inhibition in *BUB2-myc9* cells, the different abilities of Bub2R85A and Bub2-myc9 in activating the spindle position checkpoint could be explained by their different abilities to recruit Bfa1 at SPBs. In fact, whereas Bub2-myc9 is more effective than wild-type Bub2 at keeping Bfa1 at both SPBs during anaphase, Bub2R85A fails to bring Bfa1 to either SPB throughout the cell cycle. Of course, such a model does not rule out the possibility that Bub2 GAP activity helps inhibit Tem1 upon checkpoint response in wild-type cells. In any case, our data indicate that Bub2 GAP activity promotes the disappearance of the Bub2–Bfa1 complex from the mother-bound SPB at the onset of anaphase (Fig. 7 B) because both Bub2R85A and Bub2-myc9

are maintained on both SPBs from S phase to telophase. Upon spindle position checkpoint activation, Bub2 would be required to maintain Bfa1 at SPBs (Fig. 7 A), whereas its GAP activity could render the system more dynamic and help release Tem1 from Bfa1, along with Cdc5-dependent Bfa1 phosphorylation. Lte1-dependent recruitment of Tem1 on the bud-directed SPB after anaphase would also contribute to MEN activation (Fig. 7 B). Whether the in vivo target of Bub2 GAP activity in promoting its own disappearance from the mother-bound SPB is Tem1 or other proteins remains to be established. One possibility is that bud neck G proteins get exposed to Bub2 only when the daughter-directed SPB crosses the bud neck, thus signaling Bub2/Bfa1 disappearance from the mother-bound SPB. The finding that bud neck components are required for Bub2 asymmetric localization at SPBs (see the following paragraph) supports this hypothesis.

Signaling mitotic exit through the transit of one SPB through the bud neck

Inappropriate mitotic exit of spindle positioning–defective mutants often correlates with interaction of the spindle with the bud neck (Adames et al., 2001). In addition, during the unperturbed cell cycle, mitotic exit is tightly linked to the passage of one SPB through the bud neck (Molk et al., 2004). Our data clearly indicate a relationship between Bub2/Bfa1 disappearance from the mother-bound SPB and the function of bud neck components (i.e., PAK kinases; septins; and the protein kinases Hsl1, Gin4, and Swe1), thus providing a molecular basis for the aforementioned results. In fact, impairment of bud neck kinases allows Bub2, and presumably its partner Bfa1, to persist on both SPBs even when the spindle is properly oriented during anaphase. Localization of bud neck components takes place in a hierarchical manner, with PAK kinases contributing to assembly of the septin ring (Cvrckova et al., 1995), which is in turn essential for recruiting Gin4 and Hsl1 to the bud neck (Barral et al., 1999; Longtine et al., 2000), where they are required for Swe1 localization (Longtine et al., 2000). This suggests that Swe1 might promote Bub2/Bfa1 disappearance from the mother-bound SPB more directly than upstream components. However, other bud neck components beside Swe1 are likely implicated in this process because the fraction of anaphase *swe1Δ* cells with symmetrically localized Bub2 further increases upon septin ring disruption by a *cdc12* mutation or *CLA4t* overexpression. We therefore propose that passage of the daughter-directed SPB through the bud neck signals the removal of Bub2/Bfa1 from the mother-bound SPB, thus setting Tem1 free of inhibition at this spindle pole. This, together with the Lte1-mediated recruitment of additional Tem1 at the daughter-directed SPB and the Cdc5-dependent inhibition of Bfa1, perhaps taking place at the same SPB, would trigger mitotic exit (Fig. 7 B). How the signal is transmitted from the SPB passing through the bud neck to the mother-bound SPB is unclear at the moment, but the plus-end microtubule binding protein Bim1 might be implicated in the signaling, as its lack partially disrupts the asymmetric localization of Bub2. Uncovering the molecular details of this process will be an important challenge for the future and will shed light on the mechanisms coupling mitotic exit and spindle positioning in yeast as well as in other eukaryotic organisms.

Materials and methods

Strains, media, and reagents

All yeast strains (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200507162/DC1) were derivatives of or were backcrossed at least three times to W303 (*ade2-1*, *trp1-1*, *leu2-3,112*, *his3-11,15*, *ura3*, and *ssd1*). Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, and 50 mg/l adenine) supplemented with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% raffinose and 1% galactose (YEPRG). Unless otherwise stated, α factor, nocodazole, and benomyl were used at 2, 15, and 12.5 μg/ml, respectively. Synchronization experiments were performed at 25°C. For galactose induction of synchronized cells, galactose was added half an hour before release from α factor. *MET3-CDC5* cells were grown in synthetic medium lacking Met, whereas shutoff of the *MET3* promoter was done by resuspending cells in YEPD medium supplemented with 2 mM Met. Bacterial cells were grown in LD broth (1% bactotryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.25) supplemented with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol.

Plasmid constructions and genetic manipulations

Standard techniques were used for genetic manipulations (Sherman, 1991; Maniatis et al., 1992). To generate the *MET3-CDC5* plasmid (pSP82), a NdeI–XhoI PCR fragment containing *CDC5* open reading frame was cloned in the NdeI–XhoI sites of a pRS305 vector carrying the *MET3* promoter (pSP81). pSP82 integration was directed at the *LEU2* locus by EcoRI digestion. Single integration of the plasmid was assessed by Southern analysis. To clone *BUB2* under the *GAL1-10* promoter (plasmid pSP67) an HpaI–SphI PCR product containing the *BUB2* coding region was cloned into [BamHI]–SphI of a *GAL1-10*–bearing YIplac211 vector. pSP67 integration was directed to the *URA3* locus by StuI digestion. The copy number of the integrated plasmid was verified by Southern analysis. To clone *TEM1* under the *GALs* promoter (plasmid pSP233), a BamHI–SalI PCR product was ligated into BamHI–SalI of a *GALs*-bearing YCp vector (Mumberg et al., 1994). To generate a *TEM1*-containing YCp vector (pSP237), a SmaI PCR fragment bearing the *TEM1* coding region was ligated into SmaI of pFL39. To generate HA-tagged alleles, *BUB2*, *BFA1*, and *TEM1* were tagged immediately before the stop codon by one-step gene tagging (Knop et al., 1999). The *TEM1-GFP* pRS315 plasmid (Pereira et al., 2002) was a gift from E. Schiebel (Center for Molecular Biology Heidelberg, Heidelberg, Germany). The *bub2R85A* allele was produced by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene) on pSP279, a YIplac211 vector carrying 355 bp of 5′ noncoding and 663 bp of coding region of *BUB2*. Integration of the mutagenized plasmid (pSP285) was directed to the *BUB2* locus by BamHI digestion, thus generating a full-length mutant allele plus a truncated gene. Copy number of the integrated plasmid was verified by Southern analysis. To express *BUB2* in *Escherichia coli*, wild-type or R85A mutant *BUB2*, either untagged or tagged with three HA or nine myc epitopes, were amplified by PCR from genomic DNA and subcloned in the EcoRI site of pGEX6P-2rbs (a gift from A. Musacchioi, Instituto Europeo di Oncologia, Milan, Italy) to generate pSP359 (*GST-bub2R85A*), pSP295 (*GST-bub2R85A-HA3*), pSP296 (*GST-BUB2-HA3*), pSP358 (*GST-BUB2*), pSP304 (*GST-bub2R85A-myc9*), and pSP312 (*GST-BUB2-myc9*). A BamHI–PstI PCR fragment containing the *TEM1* open reading frame was cloned into BamHI–PstI of pPROEX HTa (Invitrogen) to generate pSP276. The *MBP-BFA1* construct (Pereira et al., 2002) was a gift from M. Geymonat (National Institute for Medical Research, London, UK).

Protein expression and purification

E. coli BL21 carrying pLysE plasmid (Novagen) and *TEM1-6×His*, *MBP-BFA1*, GST, GST-*BUB2*, GST-*BUB2-HA3*, GST-*BUB2-myc9*, GST-*bub2R86A*, GST-*bub2R86A-HA3*, and GST-*bub2R86A-myc9* expression plasmids were grown in LD broth containing ampicillin and chloramphenicol at 37°C for 3 h, transferred to 14°C for 1 h, and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 15 h. Cells expressing MBP-Bfa1, Tem1-6×His, and different GST-Bub2 fusion proteins were resuspended, respectively, in the following cold lysis buffers: 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM DTT, and 1 mM 4-(2-Aminoethyl)-bezenesulfonylfluoride; 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2 mM $MgCl_2$, and 10 mM imidazole supplemented with a cocktail of protease inhibitors (Complete; Boehringer); and 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 2 mM DTT supplemented with a cocktail of protease inhibitors. Cells were incubated with 1 mg/ml lysozyme in ice for 30 min, placed at 37°C for 5 min, and sonicated at 4°C. The extract was then clarified by centrifugation at 15,000 rpm for 30 min at 4°C. Tem1-6×His fusion protein was purified by

affinity chromatography with Ni-NTA columns (QIAGEN). The MBP-Bfa1 fusion protein was purified using amylose resin (New England Biolabs, Inc.), whereas the different GST-Bub2 fusion proteins were purified with glutathione-Sepharose (GE Healthcare). After elution, the fusion proteins were dialyzed against 50 mM Tris-HCl, pH 7.5, and 200 mM NaCl and stored at –80°C. For quantification, purified proteins were analyzed by Coomassie staining and by Western blot with anti-GST polyclonal antibodies (Santa Cruz Biotechnology, Inc.), anti-MBP Mab antibodies (New England Biolabs, Inc.), and 6×His Mab (CLONTECH Laboratories, Inc.).

GTPase assays

GTPase assays were performed according to Geymonat et al. (2002). In brief, 1 μg of Tem1-6×His was incubated in 25 μl of loading buffer (20 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM DTT) containing 0.1 MBq of γ-[^{32}P]GTP or 0.03 MBq of γ-[^{35}S]GTP in the absence or presence of MBP-Bfa1 for 10 min at 30°C. The reaction was then put on ice, and 10 μl of reaction were added to 50 μl of reaction buffer (20 mM Tris-HCl, pH 7.5, 2 mM GTP, and 0.6 μg/μl BSA) containing GST-Bub2. The mixture was incubated at 30°C, and for each time point 10 μl of the reaction was diluted in 990 μl of cold washing buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM $MgCl_2$). The samples were filtered through nitrocellulose filters, washed with 12 ml of cold washing buffer, and air dried, and the filter-bound radioactivity nucleotide was determined by scintillation counting. Each assay was repeated at least two times, and reproducible results were obtained.

Immunoprecipitations, Western blot analysis, and kinase assays

Immunoprecipitations were performed as described in Fraschini et al. (2001). Bub2-myc9 was immunoprecipitated from 1 mg of total extract by protein A–Sepharose beads cross-linked to anti-myc antibodies. Bub2-HA3 and Bfa1-HA6 were immunoprecipitated from 1 mg of total extract by protein A–Sepharose beads cross-linked to anti-HA antibodies. For Western blot analysis, protein extracts were prepared according to Surana et al. (1993). Proteins transferred to Protran membranes (Schleicher & Schuell) were probed with 9E10 mAb for myc-tagged Bub2, with 12CA5 mAb for HA-tagged Bub2 and Bfa1, and with polyclonal antibodies against GFP for GFP-tagged Tem1 (Invitrogen), Swi6, Clb2, and Sic1. Secondary antibodies were purchased from GE Healthcare, and proteins were detected by an enhanced chemiluminescence system according to the manufacturer. Histone H1 kinase activity was measured as previously described (Surana et al., 1993).

Other techniques

Flow cytometric DNA quantitation was determined according to Fraschini et al. (1999) on a FACScan (Becton Dickinson). In situ immunofluorescence was performed according to Fraschini et al. (1999). Immunostaining of α-tubulin was performed with the YOL34 monoclonal antibody (Serotec) followed by indirect immunofluorescence using rhodamine-conjugated anti–rat antibody (1:100; Pierce Chemical Co.). Immunostaining of myc-tagged proteins was done with the 9E10 mAb, whereas that of HA-tagged proteins was done with the 16B12 mAb (Babco), followed by indirect immunofluorescence using CY3-conjugated goat anti–mouse antibody (1:1,000; GE Healthcare). Cytokinesis defects were assessed upon cell wall digestion with zymolase. Digital images were acquired on a fluorescent microscope (Eclipse E600; Nikon) equipped with a charge-coupled device camera (DC350F; Leica) at 20°C with an oil 100× 1.3 NA Plan-Fluor objective (Nikon), using FW4000 software (Leica).

Online supplemental material

Fig. S1 shows that overexpression of *BUB2* in *cdc5-2* cells causes effects similar to Bub2-myc9. Fig. S2 shows that symmetrically localized Bub2 prevents exit from mitosis in *esp1-1* cells. Fig. S3 demonstrates that Bub2 does not stimulate GTP dissociation from Tem1. Fig. S4 shows that the GAP-defective *bub2R85A* mutant fails to activate the spindle position checkpoint. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200507162/DC1.

We wish to thank M. Geymonat, T. Huffaker, T. Hyman, D. Kellogg, J. Kilmartin, K. Lee, A. Musacchio, M. Muzi Falconi, K. Nasmyth, M. Rose, and E. Schiebel for strains and plasmids; M. Tyers and W. Zachariae for antibodies; S. Sabbioneda and M. Muzi-Falconi for technical advice on protein purification; L. Merlini for help with GAP assays; and L. Dirick, M.P. Longhese, A. Musacchio, E. Schwob, and members of our laboratory for useful discussions and critical reading of the manuscript.

This work was supported by grants from the Associazione Italiana Ricerca sul Cancro to S. Piatti and from Cofinanziamento 2003 Ministero dell'Università e della Ricerca Scientificae Technologica, Università di Milano-Bicocca and Fondo per gli investimenti della Ricerca di Base to G. Lucchini.

Submitted: 28 July 2005
Accepted: 27 December 2005

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