Title

A novel role for the CBF3 kinetochore-scaffold complex in regulating septin dynamics and cytokinesis

Journal

Journal of Cell Biology, 171(5)

ISSN

0021-9525

Authors

Gillis, Amethyst N
Thomas, Scott
Hansen, Scott D
et al.

Publication Date

2005-12-01

Peer reviewed

A novel role for the CBF3 kinetochore–scaffold complex in regulating septin dynamics and cytokinesis

Amethyst N. Gillis, Scott Thomas, Scott D. Hansen, and Kenneth B. Kaplan

The Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA 95616

In budding yeast, the kinetochore scaffold complex centromere binding factor 3 (CBF3) is required to form kinetochores on centromere DNA and to allow proper chromosome segregation. We have previously shown that *SKP1* and *SGT1* balance the assembly and turnover of CBF3 complexes, a cycle that we suggest is independent of its role in chromosome segregation (Rodrigo-Brenni, M.C., S. Thomas, D.C. Bouck, and K.B. Kaplan. 2004. *Mol. Biol. Cell.* 15:3366–3378). We provide evidence that this cycle contributes to a second, kinetochore-independent function of CBF3. In this study, we show that inhibiting the assembly of CBF3 causes disorganized septins and defects in cell polarity that give rise to cytokinesis failures. Specifically, we show that septin ring separation and disassembly is delayed in anaphase, suggesting that CBF3 regulates septin dynamics. Only mutations that affect the CBF3 cycle, and not mutants in outer kinetochore subunits, cause defects in septins. These results demonstrate a novel role for CBF3 in regulating cytokinesis, a role that is reminiscent of passenger proteins. Consistent with this possibility, we find that CBF3 interacts with Bir1p, the homologue of the passenger protein Survivin. Mutants in Bir1p similarly affect septin organization, leading us to propose that CBF3 and Bir1p act as passenger proteins to coordinate chromosome segregation with cytokinesis.

Introduction

Kinetochores consist of multiple discrete protein complexes that are assembled in a hierarchical fashion on centromeric (CEN) DNA (for review see McAinsh et al., 2003). Kinetochore formation is nucleated by a DNA protein scaffold. In animal cells, this scaffold is believed to consist of specialized nucleosomes containing the histone H3 variant CENP-A (centromere protein A). In the budding yeast *Saccharomyces cerevisiae*, a sequence-specific DNA-binding complex, centromere binding factor 3 (CBF3), is required in addition to nucleosomes containing CENP-A (Cse4p in yeast). The absolute dependence of yeast kinetochores on CBF3 has led to the well accepted view that it establishes a nucleation site required for all other kinetochore proteins to associate with the centromere.

CBF3 consists of multiple subunits; the Ctf13p subunit forms the core of the complex, physically contacting both Cep3p and Ndc10p and, together, making direct contacts with CEN DNA (Espelin et al., 1997; Kaplan et al., 1997; Russell et al., 1999; Rodrigo-Brenni et al., 2004). Assembly of this complex is necessary for binding CEN DNA and requires Skp1p, Sgt1p, and the HSP90 chaperone (Kitagawa et al., 1999; Stemmann et al., 2002; Bansal et al., 2004; Lingelbach and Kaplan, 2004). The assembly of CBF3 is balanced by its turnover; Ctf13p is targeted for ubiquitin-mediated degradation and CBF3 complexes are themselves unstable (Lingelbach and Kaplan, 2004; Rodrigo-Brenni et al., 2004). We refer to this balance of assembly and turnover as the CBF3 cycle, and we have shown that this cycle occurs throughout the cell division cycle. Inhibiting CBF3 assembly does not disrupt kinetochores on CEN DNA, neither by GFP fusion localization nor by chromatin immunoprecipitation (Rodrigo-Brenni et al., 2004). Our interpretation of these results is that CBF3 complexes are stably bound to CEN DNA and that the CBF3 cycle may contribute to a noncentromeric role for CBF3.

Although kinetochore assembly on CEN DNA leads to the formation of a microtubule attachment site, the dynamic properties of kinetochores have been associated with mitotic progression. For example, spindle checkpoint proteins associate with kinetochores early in mitosis and are down-regulated after chromosomes have formed proper bivalent attachments with the mitotic spindle (Cleveland et al., 2003; Lew and Burke, 2003). In anaphase, the coordination of chromosome segregation with the start of cytokinesis is associated with the transfer of passenger proteins from kinetochores to the central spindle (Vagnarelli and Earnshaw, 2004; Yang et al., 2004).

Correspondence to Kenneth B. Kaplan: kbkaplan@ucdavis.edu

Abbreviations used in this paper: CBF3, centromere binding factor 3; CEN, centromeric; DIC, differential interference contrast; PP2A, protein phosphatase type 2A complex; SCF, Skp1-cullin-F-box.

This online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 171, No. 5, December 5, 2005 773–784
http://www.jcb.org/cgi/doi/10.1083/jcb.200507017

Supplemental Material can be found at:
http://www.jcb.org/cgi/content/full/jcb.200507017/DC1

The association of passenger complexes with kinetochores has been proposed to allow the proper position of these proteins on the central spindle during anaphase. However, a wealth of evidence now indicates that these proteins also play an important role in chromosome congression and in resolving misoriented kinetochore spindle attachments (Petersen et al., 2001; Tanaka et al., 2002; Lampson et al., 2004). How these early mitotic roles relate to the function of passenger proteins in cytokinesis is unclear. Inhibition studies have shown that the passenger proteins Aurora B, INCENP (inner centromere protein), and Survivin are required for cytokinesis in animal cells (Terada et al., 1998; Kaitna et al., 2000; Terada, 2001; Murata-Hori et al., 2002; Yang et al., 2004), but it is not known exactly how these proteins regulate the cytokinetic apparatus. In yeast, it is unknown if the passenger protein homologues Ipl1p, Sli15p, and Bir1p also have a role in cytokinesis. Recent studies have shown that Ipl1p and Sli15p localize to interpolar microtubules and regulate spindle stability in anaphase (Buvelot et al., 2003; Pereira and Schiebel, 2003). Interestingly, the CBF3 mutant *ndc10-1* similarly affects spindle stability and compromises cytokinesis, suggesting that kinetochore complexes may participate in regulating multiple anaphase events (Ducat and Zheng, 2004; Bouck and Bloom, 2005).

We show that the kinetochore scaffold complex CBF3 has an unexpected second role in regulating septins and cytokinesis. Several lines of evidence suggest that this second role is independent of its function in forming kinetochores and segregating chromosomes: (a) only CBF3 mutants, but not mutants in outer kinetochore complexes, affect septin organization; (b) *SKP1* and *SGT1* alleles that affect CBF3 turnover, but not chromosome segregation, compromise septin organization; and (c) inhibiting CBF3 in cells arrested in G1 with mating pheremones also causes defects in septin organization. Measurements of septin dynamics in anaphase reveal that the CBF3 cycle is required for proper ring separation and disassembly of the mother septin ring; these defects contribute to aberrant polarized cell growth, bud site selection, and cytokinesis. Mutants in the passenger protein homologue *BIR1*, but not in *IPL1* or *SLI15*, also result in septin organization defects. These results lead us to propose that CBF3 and Bir1p function as a passenger complex that regulates septins and cytokinesis.

Results

Inhibiting CBF3 assembly compromises polarized cell growth

To investigate whether the cycle of CBF3 assembly and turnover plays a role in mitotic progression independent of chromosome segregation, we examined the consequences of inhibiting CBF3 assembly in dividing yeast cells. We used a haploid strain of yeast containing a *GAL1* promoter that was integrated in place of the normal *CTF13* promoter (*GAL1-CTF13*). Ctf13p is the core of the CBF3 complex and is targeted for ubiquitin-mediated degradation (Kaplan and Sorger, 1997). Growth of this strain in the presence of dextrose or raffinose results in the rapid depletion of Ctf13p ($t_{1/2}$ = 10 min) and CBF3 complexes ($t_{1/2}$ = 25 min) as measured in cell extracts (Rodrigo-Brenni et al., 2004). As previously reported, inhibition of CBF3 assembly does not compromise the integrity of kinetochores as judged by GFP-kinetochore markers and chromatin immunoprecipitation experiments, supporting the idea that CEN-associated CBF3 is not rapidly turned over (Rodrigo-Brenni et al., 2004). Consistent with this observation, cells inhibited for CBF3 assembly only transiently arrest in metaphase and segregate their chromosomes (unpublished data). After the growth of these cells in raffinose to inhibit CBF3 assembly, we observed that 30–60% of cells had elongated buds (Fig. 1, A and B; and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200507017/DC1) with concentrated patches of actin at their bud tips, which is consistent with failure to switch from axial to isotropic bud growth (Fig. 1 A). In contrast, the parent strain containing the wild-type *CTF13* promoter did not show a significant number of cells with elongated buds and exhibited a normal pattern of actin staining (Fig. S1 B and not depicted). Overexpression of Ctf13p (galactose) also increases the percentage of cells with elongated buds, although to a lesser extent than the inhibition of CBF3 assembly (10–14% compared with 2% in wild-type cells; Figs. 1 B and S1 B). Deletion of *SWE1*, a negative regulator of CDK–cyclin complexes, favors early isotropic growth and frequently rescues elongated buds in polarization mutants (Richman et al., 1999). We found that this was also the case in cells inhibited for CBF3 assembly; the deletion of *SWE1* eliminates the appearance of elongated buds and restores the isotropic distribution of actin in the bud, even after 5 h of growth in raffinose (Fig. 1, A and B). In addition to the patches of actin at the bud tips, we also observed that actin failed to properly accumulate at the mother bud neck in anaphase cells inhibited for CBF3 assembly; actin was often found in disorganized patches positioned away from the mother bud neck of anaphase cells (Fig. 1 C, arrows). In contrast to the hyperpolarized bud growth, however, deletion of *SWE1* did not rescue the mislocalized actin patches in anaphase cells (Fig. 1, C and D). Together, these data suggest that perturbing the CBF3 cycle compromises the ability of wild-type cells to properly position actin, either at bud tips or at the site of cell division.

CBF3 is required to organize septins

Defects in actin redistribution are often associated with changes in the underlying septin scaffold. Septins form a filamentous structure at the site of bud growth and at the mother bud neck and have been shown to be important for both bud growth and cytokinesis (Longtine and Bi, 2003). Therefore, we examined the possibility that altering the CBF3 cycle compromises septins. Wild-type control and *GAL1-CTF13* strains were grown in galactose or raffinose, and cells were fixed and stained with antibodies to the septin Cdc11p (Mortensen et al., 2002). In control cells, septins form a ring at the incipient bud site and a double ring in small or large budded cells grown in either raffinose or galactose (Fig. 1 E, *CTF13*). In the *GAL1-CTF13* strain, the overproduction of Ctf13p resulted in a modest but significant increase in the percentage of cells that had abnormal septin organization (29 vs. 7% in control strains; Fig. 1 E [*GAL1-CTF13*, galactose] and Fig. S1 C). The percentage of cells with disorganized septins dramatically increased when

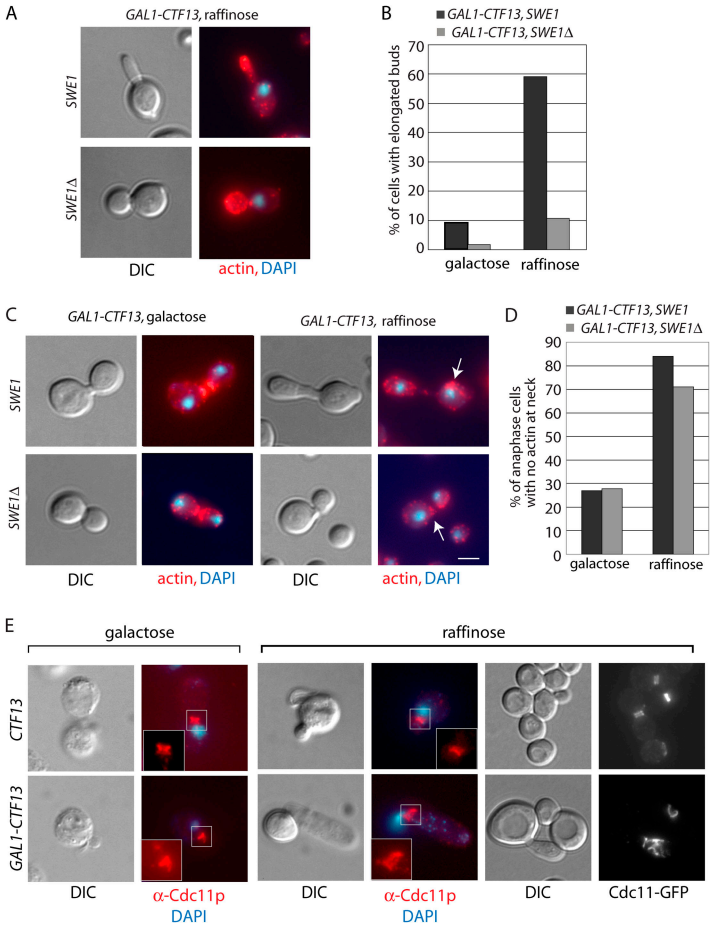

Figure 1. **Inhibition of CBF3 assembly causes defects in polarized cell growth and septin organization.** *CTF13* or *GAL1-CTF13* strains were grown in the presence of galactose and switched to fresh medium containing either galactose or raffinose for 300 min. Samples were collected, fixed, and stained with phalloidin coupled to Texas red to visualize actin (red) and with DAPI to visualize chromosomes (blue). *GAL1-CTF13* strains with either *SWE1* or *SWE1Δ*, a single mass of chromosomes, and elongated buds (A) were recorded and the percentage of cells with elongated buds was calculated for each condition (B). (C) Anaphase cells from the aforementioned strains were recorded, and the percentage of anaphase cells that failed to concentrate actin at the mother bud neck was calculated (D). Arrows indicate aberrantly localized actin patches in anaphase cells. (E) *CTF13* or *GAL1-CTF13* strains were grown in the indicated medium for 120 min, fixed, and processed for immunofluorescence with antibodies to Cdc11p (red). Chromosomes were stained with DAPI (blue). Inset represents a 1.5-fold zoom of the septin staining. The indicated strains expressing Cdc11-GFP from its endogenous promoter (Table I) were grown in raffinose for 120 min and photographed in DIC and fluorescent channels. Bar, 1 μm.

this strain was grown in raffinose to inhibit CBF3 assembly (Fig. S1 C). A similar pattern of septin disorganization was observed in strains carrying a Cdc11-GFP fusion and appeared to be more severe than in untagged strains (Fig. 1 E, Fig. S1 E, and not depicted). We speculate that the GFP fusion may partially compromise Cdc11p function, although it appears normal in control cells (Fig. 1 E, *CTF13*), and exacerbate the septin defect in cells when CBF3 assembly has been inhibited.

Disorganized septins may reflect a failure to properly regulate their dynamics during the cell cycle. In addition, we observed the most severe septin defects in anaphase cells (Fig. S1 D). To examine the requirement for CBF3 assembly during anaphase more directly, we arrested strains that contain Cdc11-GFP and either *CTF13* or *GAL1-CTF13* in S phase, using hydroxyurea. Cells were transferred to media containing raffinose for 2 h in the presence of hydroxyurea (Fig. 2 A). We observed no change in septin organization after the hydroxyurea arrest in either control or *GAL1-CTF13* strains (Fig. 2 B, +raffinose HU arrest). Cells were released from S phase by washing out the hydroxyurea and returning them to medium containing raffinose with or without nocodazole, a microtubule poison that arrests cells in metaphase. In cells arrested in metaphase, inhibition of CBF3 assembly had no effect on the Cdc11-GFP pattern (Fig. 2 B, +raffinose, HU release, +nocodazole). Finally, we released cells from the hydroxyurea-arrest into medium containing raffinose and allowed them to progress past metaphase; fixing and staining cells indicated that >90% of large budded cells were in anaphase under these conditions (unpublished data). In control cells, we observed the normal separation of septin rings marking the beginning of anaphase. In contrast, a high percentage of cells inhibited for CBF3 assembly exhibited disorganized septins during this same time period (Fig. 2, B [+raffinose, HU release] and C). From these data, we concluded that CBF3 assembly is critical for septin organization after the movement of chromosomes to the poles in anaphase, at a time when septins are separating to form the cytokinetic furrow.

CBF3 is required for septin ring separation and disassembly in anaphase

To more directly address how CBF3 assembly regulates anaphase septins, we filmed the behavior of Cdc11-GFP in mitotic cells. In wild-type cells, septins switch from a filamentous structure in G2 to distinct rings as mitosis begins (compare Fig. 3 [G2] with Fig. S1; also compare HU arrest with HU release in Fig. 2 B). Septin rings undergo further separation 15–45 min after they become distinct during anaphase (Fig. 3 A, separation II; shortly [2–6 min] after septins are maximally separated, the septin ring in the mother cell undergoes disassembly [Fig. 3 A, disassembly]); cells finish cytokinesis and rapidly return to G1 as indicated by the reformation of the septin ring in the mother cell (7–13 min after disassembly; Fig. 3 A, reformation).

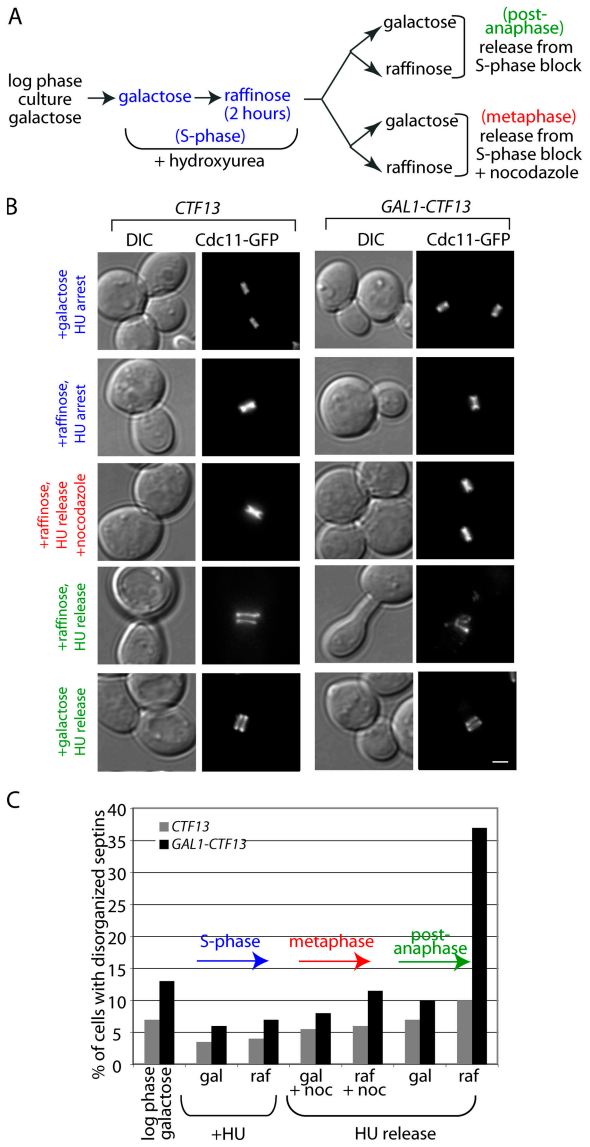

Figure 2. **Septin organization requires CBF3 assembly during anaphase.** (A) The indicated strains were grown as outlined in the flow chart. (B and C) Stages where cells were removed for analysis are indicated by colors (blue, S phase; red, metaphase; and green, postanaphase cells). (B) DIC and fluorescent images of Cdc11-GFP were collected as indicated in A. (C) The total percentage of cells with disorganized septins was calculated. Bar, 1 μm.

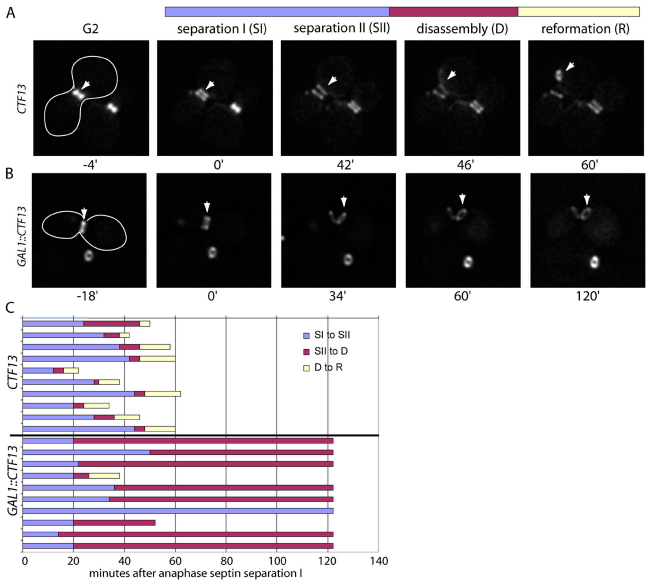

Figure 3. **Inhibition of CBF3 assembly causes defects in septin ring separation and disassembly.** After growth in raffinose for 120 min, large-budded cells expressing Cdc11-GFP and containing *CTF13* (A) or *GAL1-CTF13* (B) were selected for recording. Panels depict the major transitions in septin behavior for *CTF13* or *GAL1-CTF13* strains (see Videos 1–3 for the complete sequence, available at http://www.jcb.org/cgi/content/full/jcb.200507017/DC1). (C) Time 0 represents the first appearance of distinct septin rings; the transitions are defined as follows and the length of time for each transition is plotted for multiple cells. The violet column indicates the time between SI and SII or when septin ring separation is complete. The magenta column represents the time between SII and the disassembly of the septin ring in the mother cell (D). The yellow column represents time between disassembly (D) and reformation of a new septin ring in the mother cell (R).

The general timing of anaphase as judged by septin behavior is remarkably consistent between cells with very similar transitions between each septin stage (Fig. 3 C, *CTF13*; and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200507017/DC1). In cells where CBF3 assembly is inhibited (Fig. 3 B, *GAL1-CTF13*), septin rings become distinct in large budded cells but show more variable kinetics during the transition from SI to SII (wild type = 32 ± 11 min; *GAL1-CTF13* = 48 ± 42 min; Fig. 3 C). More dramatically, separation of rings occurs asymmetrically in the majority of cells inhibited for CBF3 assembly (92% in CBF3-inhibited cells compared with 0.2% in wild-type cells); in these cells, one portion of the ring in the mother cell remains in close proximity to the daughter cell ring (Fig. 3 B, 34-min time point). Finally, after this aberrant ring separation we observed that the mother cell ring is dramatically delayed for disassembly (Fig. 3, B and C; and Video 2). In most cases, the ring fails to disassemble during the time course of the experiment (mean >70 min) in contrast to the rapid disassembly observed in wild-type cells (mean = 10 min). Remarkably, we also observed several unbudded (G1) cells where the septin ring disassembled and then reformed in a nonaxial position, suggesting that the timing of septin dynamics is misregulated in cells inhibited for CBF3 assembly (Video 3). Together, these data argue that the CBF3 cycle regulates the timing of septin dynamics during the anaphase and G1, possibly through changes in their posttranslation modifications.

Our observations that unbudded cells (G1) undergo inappropriate septin ring disassembly and reformation led us to ask if CBF3 can regulate septins independent of chromosome segregation and mitosis. As in anaphase, septins also undergo a dramatic reorganization during the mating process. After α factor treatment, haploid cells reorganize their septins into perpendicular (with respect to the bud neck) arrays during formation of the "shmoo" mating projection (Fig. 4 B, *CTF13*; Ford and Pringle, 1991; Kim et al., 1991; Longtine et al., 1998). To determine if the CBF3 cycle is important for the reorganization of septins in the shmoo, we treated *GAL1-CTF13* or control cells with α factor and examined the distribution of Cdc11-GFP. In the *GAL1-CTF13* strain, elevated (galactose) or reduced (raffinose) CBF3

levels resulted in less organized septins (Fig. 4 B, arrowheads) in the shmoo tip compared with the control strain. When α factor is removed, cells reinitiate the budding process and septins form the parallel structures typically observed in cycling cells. We released α factor–arrested cells into hydroxyurea to prevent them from entering mitosis and monitored Cdc11-GFP in the presence or absence of CBF3 assembly (Fig. 4 A). When released into media containing galactose, both *GAL1-CTF13* and control cells exhibited well organized parallel septin rings at the mother bud neck (Fig. 4, B [arrows] and C). In contrast, when cells were released from the α factor arrest and CBF3 assembly was inhibited, septins became dramatically disorganized. The shape of the resulting bud also indicated that the morphological transition between the shmoo and bud had been compromised (Fig. 4 B, compare differential interference contrast [DIC] images). These results indicate that CBF3 is required to regulate septins during the G1 stage of the cell cycle, supporting the idea that the function of CBF3 in organizing septins is distinct from its role in segregating chromosomes (see Discussion).

CBF3 regulates septins independent of its role in chromosome segregation

Although kinetochores remain intact and anaphase chromosome segregation occurs normally after the depletion of CBF3 (Rodrigo-Brenni et al., 2004), it is possible that there are subtle changes in outer kinetochore complexes that affect septin organization. To test this possibility, we examined septins in a variety of mutants that compromise either CBF3 or outer kinetochore complexes. We observed little change in septin organization in mutants that effect outer kinetochore complexes (e.g., *ndc80-1*; Fig. 5, A and B). Although some outer kinetochore alleles arrest in metaphase because of the spindle checkpoint, we and others have observed that some alleles are checkpoint defective and continue past metaphase (e.g., *ctf19-26, ctf19-58, ndc80-1*, and *dam1-1*; Hyland et al., 1999; Jones et al., 2001; Wigge and Kilmartin, 2001), making it unlikely that a pre-anaphase arrest masks a septin defect. In contrast, alleles that affect CBF3 assembly exhibit a high percentage of cells with disorganized septins (Fig. 5 A and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200507017/DC1). As was the case for the *GAL1-CTF13* strain, we observed partially formed or misoriented septin rings, especially in anaphase cells. Interestingly, the most dramatic affects on septins were observed in *SKP1* and *SGT1* alleles that we have previously shown compromise the CBF3 cycle (Fig. 5 B; Lingelbach and Kaplan, 2004; Rodrigo-Brenni et al., 2004). Specifically, the *skp1-4* allele, which prevents CBF3 assembly by blocking the interaction between Skp1p and Sgt1p, gives rise to defects in septin organization. The *skp1-3* allele, which stabilizes the Skp1p–Sgt1p interaction and prevents CBF3 turnover (Rodrigo-Brenni et al., 2004), results in an even higher percentage of cells with disorganized septins. *SKP1* and *SGT1* have also been implicated in the function of the Skp1-cullin-F-box (SCF)–E3 ubiquitin ligase complex. To rule out a role for SCF in regulating septin organization, we examined *cdc34-2*, which is a conditional allele encoding the ubiquitin-ligase subunit of SCF. We observed no effect on septin organization despite the high percentage of cells with

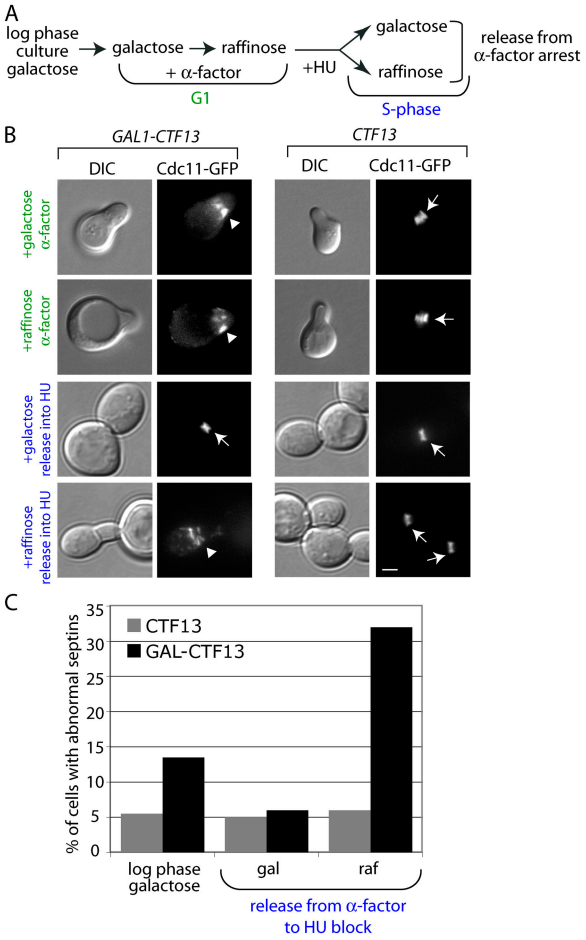

Figure 4. **Septin organization in shmoos requires CBF3 assembly.** (A) The indicated strains were grown as outlined in the flow chart. (B) Stages where cells were removed for analysis are indicated by colors (green, α factor–induced arrest in G1; blue, S phase arrest in hydroxyurea [HU]). (B) DIC and fluorescent images of Cdc11-GFP were collected as indicated in A. The arrows indicate normal septin organization; arrowheads indicate cells with disorganized septins. (C) The total percentage of cells with disorganized septins was calculated. Bar, 1 μm.

elongated buds (Fig. 5, A and B). In contrast to the more subtle effects of *SKP1* and *SGT1* alleles, the *ndc10-1* allele entirely eliminates kinetochore formation on CEN DNA and cells inappropriately proceed through the cell cycle (Gardner et al., 2001); the resulting multibudded *ndc10-1* cells frequently exhibit a complete failure to form septins rings (38% for *ndc10-1* and 5% for the wild-type W303 strain) and are therefore not included in our analysis of "disorganized" septins. This phenotype is similar to that recently shown by Bouck and Bloom (2005). However, we note that this extreme phenotype is not observed when *ndc10-1* is backcrossed to the S288C background used in all of the other data in our studies, instead we observe a pattern of septin disorganization more typical of CBF3 mutants (Fig. 5 B and Fig. S2; see Discussion). Thus, even the most severe kinetochore mutant available produces the same septin defect observed in mutants, which perturbs the CBF3 cycle but not kinetochore formation. Together, these results strongly argue that CBF3 functions independently from its role at the kinetochore to regulate septins during anaphase.

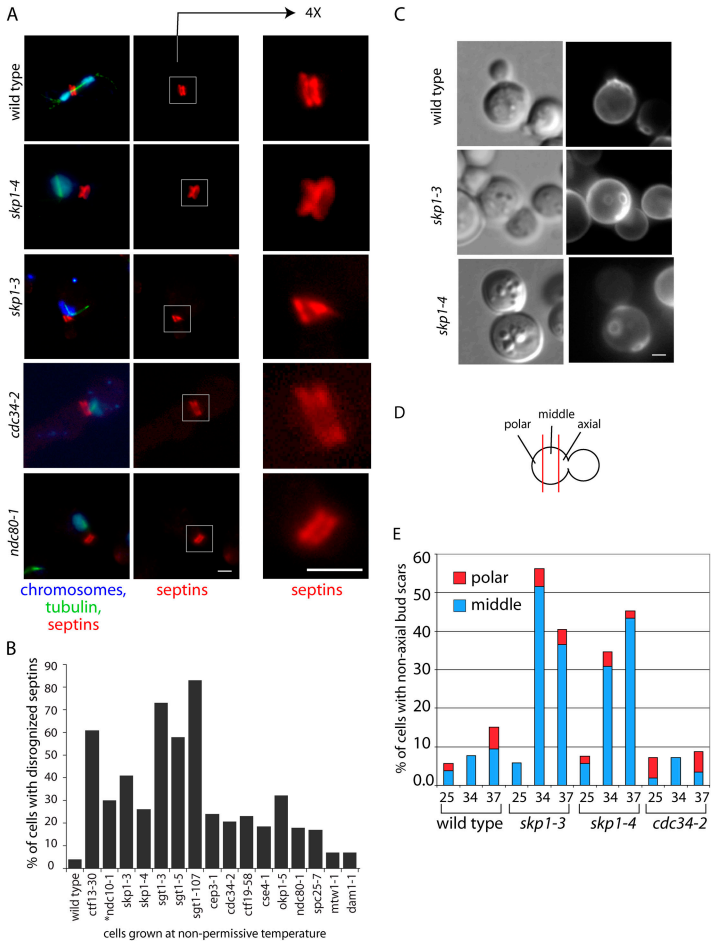

Figure 5. **Defects in CBF3 assembly, but not outer kinetochore proteins, cause septin organization defects.** (A) Representative examples of wild-type or mutant strains grown for 3 h at nonpermissive temperature, fixed, and processed to stain septins (red), tubulin (green), and chromosomes (blue). The right panels represent a fourfold zoom of the regions in the insets. (B) Quantification of the percentage of cells with defective septins after growth at nonpermissive temperature. (C) The indicated mutants were grown at nonpermissive temperature for 3 h and stained with Calcofluor white to visualize bud scars; the position of bud scars were classified as axial, middle, or polar. (D) Images were recorded using DIC and fluorescent optics. (E) Cells with more than one bud scar were scored and the percentage of total cells with scars in the middle region of the mother (blue) or at the polar side of the mother (red) was measured. Bars, 1 μm.

CBF3 organization of septins is required for proper polarity and cytokinesis

To assess the significance of the disorganized septins in CBF3 mutants, we asked whether these changes compromise bud site selection, a septin-dependent process not affected by defects in actin polarization (Richman et al., 1999). Normally, haploid yeast forms a new bud at a position adjacent to the previous daughter. This pattern of axial budding can be monitored by using the fluorescent dye Calcofluor white to visualize bud "scars" that remain on mothers after multiple rounds of division. Previous studies have demonstrated that mutations in septins give rise to random bud scar patterns (Flescher et al., 1993). In wild-type haploid cells, we observed that the majority of mother cells (85–90%) with bud scars exhibited an axial arrangement as predicted, regardless of growth temperature (Fig. 5, C and E, wild type). Although normal axial bud scars were observed under permissive conditions (25°C), we found a significant increase in the percentage of cells with random bud scar patterns in both *skp1-3* and *skp1-4* strains grown under semi- or nonpermissive conditions (34 or 37°C; Fig. 5 E). The increase in random bud scar patterns was independent of elongated bud growth, suggesting that the failure to properly position new buds is because of a defect in septins and not bud morphology. A similar phenotype was observed when CBF3 assembly was inhibited in the *GAL1-CTF13* strain (unpublished data). In contrast, mutants in outer kinetochore subunits or in the ubiquitin ligase *CDC34* do not show an increase in nonaxial bud patterns (Fig. 5 E and not depicted). Together, these results suggest that the changes in septin organization observed in CBF3 mutants compromise the ability of cells to properly control polarized bud site selection.

Septins are used in yeast throughout the cell cycle to control bud growth as well as cytokinesis. To examine whether the defects in septin organization compromise cytokinesis, we monitored the *GAL1-CTF13* strain when grown over longer periods of time in either galactose or raffinose. When CBF3 assembly was inhibited (raffinose), we observed an increase in the number of cells with segregated DNA masses and extended anaphase spindles, which is consistent with a defect in cytokinesis (Fig. 6 A). In previous studies, it has been shown that compromised cytokinesis can give rise to multinucleated cells because of defects in septins when the CDK/cyclin regulator *SWE1* is deleted (Sreenivasan and Kellogg, 1999). We examined the appearance of multinucleated anaphase cells in a *GAL1-CTF13*, *SWE1Δ* strain. We observed a small percentage of multinucleated cells when CBF3 assembly was inhibited in a wild-type *SWE1* strain and a dramatic increase in multinucleated cells when *SWE1* was deleted (9–52% of anaphase cells; Fig. 6, B and C). The accumulation of anaphase cells with aberrant septins and the increase in multinucleated cells strongly argue that CBF3 assembly is critical for cytokinesis.

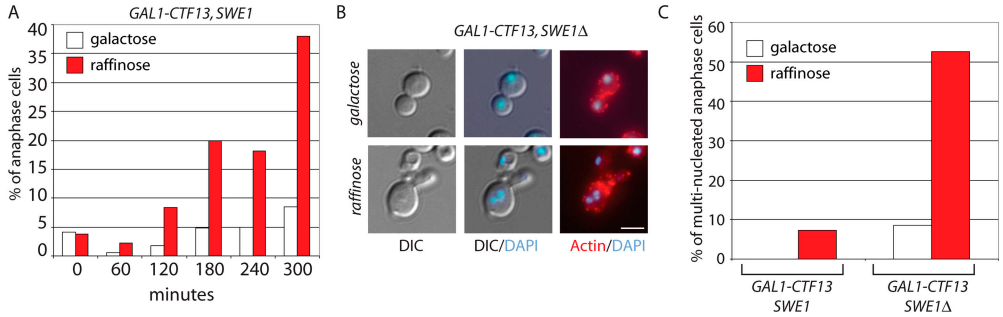

Figure 6. **Cytokinesis is compromised in cells inhibited for CBF3 assembly.** (A) *GAL1-CTF13* strains were grown in the indicated medium for 300 min, and the percentage of anaphase cells in the population was calculated after DAPI and tubulin staining (not depicted). (B) A *GAL1-CTF13, SWE1Δ* strain was grown in medium containing galactose or raffinose, cells were stained for actin (red), and chromosomes were visualized with DAPI. (C) Multinucleated cells were calculated as a percentage of the total anaphase cells in the population with more than two chromosome masses. Bar, 1 μm.

CBF3 and *BIR1* mutants specifically compromise septins

How does the CBF3 cycle alter septin behavior? The inability of CBF3 subunits to obviously interact with septins raises the possibility that CBF3 regulates septins indirectly (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200507017/DC1; and not depicted). Although several regulatory enzymes can influence septin function, the Rts1p subunit of the protein phosphatase type 2A complex (PP2A) is particularly intriguing because it transiently localizes to kinetochores and then to septins late in mitosis. In addition, a deletion of *RTS1* gives rise to septin defects in mitosis (Dobbelaere et al., 2003). To test the possibility that CBF3 and the Rts1–PP2A complex work in the same pathway to regulate septins, we carefully compared their respective septin phenotypes. Unlike our results with CBF3 mutants, septins in *RTS1*Δ did not exhibit any defects when stained with antibodies against Cdc11p or when Cdc11-GFP was visualized (Fig. S3 B). In contrast to Cdc11p and consistent with a previous study (Dobbelaere et al., 2003), we observed the appearance of multiple septin rings in the mother bud when Cdc12-GFP was expressed in wild-type cells (8%) and when *RTS1*Δ was deleted (18%); we never observed this phenotype in CBF3 mutants, further distinguishing the septin defects in *RTS1* and CBF3 mutants (Fig. 1, Fig. S1, and not depicted). We next considered the possibility that the Rts1–PP2A complex and CBF3 work in parallel pathways to regulate septins. In this case, we predict that a strain with mutations in both CBF3 and *RTS1* will result in a more severe defect in septins and cell polarity. To test this prediction, we crossed a *GAL1-CTF13* strain with a *RTS1*Δ strain and isolated several meiotic products containing both mutations. Even when grown on galactose, the double mutants (*GAL1-CTF13* and *RTS1*Δ) grew very slowly and exhibited an increase in cells with elongated buds (Fig. S3 A). Growth on raffinose to inhibit CBF3 assembly resulted in rapid cell lysis (unpublished data). Together, these results argue that CBF3 and the Rts1–PP2A complex independently regulate septin behavior.

The requirement of CBF3 to regulate septins during anaphase is reminiscent of the role ascribed to passenger proteins in higher eukaryotes. Interestingly, we and others have observed that CBF3 subunits are found both at kinetochores and on interpolar microtubules during anaphase (Fig. S3, Ndc10-GFP), a localization change that is similar to passenger protein behavior in animal cells (Buvelot et al., 2003; Pereira and Schiebel, 2003). Furthermore, in yeast *BIR1* (Survivin), *IPL1* (Aurora B) and *SLI15* (INCENP) encode likely homologues of known passenger proteins, and both Ipl1p and Sli15p have been shown to localize to interpolar microtubules late in anaphase (Buvelot et al., 2003; Pereira and Schiebel, 2003). We have confirmed these localizations (not depicted), and we now show that Bir1-GFP also localizes to kinetochores early in the cell cycle and is found on interpolar microtubules in anaphase (Fig. 7 A). Previous work identified that the CBF3 subunit, Ndc10p, interacts with Bir1p via yeast two hybrid

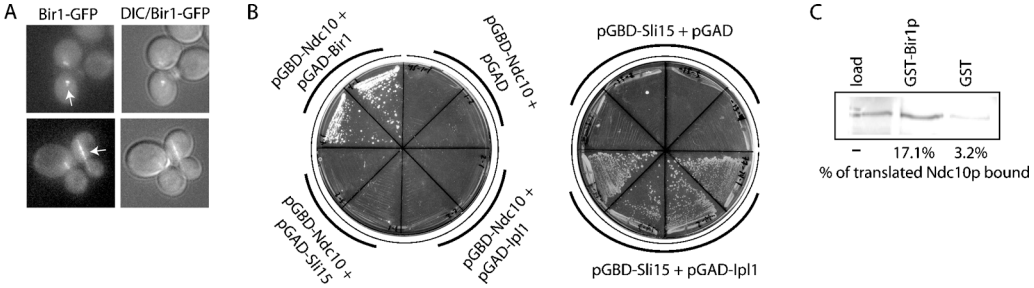

Figure 7. **Bir1p localizes to interpolar microtubules and interacts with Ndc10p.** (A) Bir1-GFP was imaged in premetaphase cells (top, arrow) and in anaphase cells (bottom, arrow); fluorescence and DIC images were recorded. (B) The indicated bait and prey yeast two-hybrid vectors were plated on HIS selection; growth indicates an interaction. (C) Ndc10p was translated in vitro in the presence of ^{35}S-methionine. The translated protein was incubated with GST-Bir1p or GST and the fraction of the Ndc10p bound to the beads was calculated.

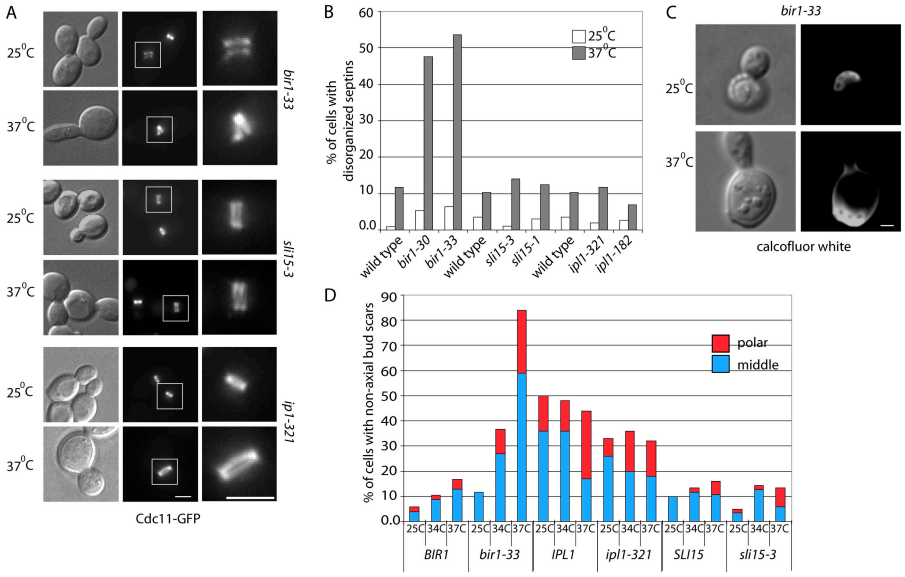

Figure 8. **Bir1p is required for septin organization.** (A) The indicated mutants expressing Cdc11-GFP were grown at permissive (25°C) and nonpermissive temperature (37°C) for 3 h; fluorescence and DIC images were recorded. The boxed regions indicate the areas on the right. (B) The percentages of cells with disorganized septins were calculated for mutants and isogenic wild-type parents grown at permissive (white bars) or nonpermissive (gray bars) temperatures. (C) The indicated mutants were grown at nonpermissive temperatures, stained with Calcofluor white to visualize bud scars and the position of the bud scars were classified as in Fig. 3 C. (D) Images were recorded and the percentage of cells with nonaxial bud scars were determined as in Fig. 3 D. Bar, 1 μm.

(Yoon and Carbon, 1999). We confirmed this result and showed that neither Ipl1p nor Sli15p interact with Ndc10p by yeast two hybrid (Fig. 7 B). To show that the interaction between Ndc10p and Bir1p is direct, we translated Ndc10p in vitro and incubated it with GST or a GST-Bir1p fusion. The enrichment of translated Bir1p with GST-Bir1p but not GST is consistent with the direct interaction between these two proteins (Fig. 7 C), indicating that CBF3 and Bir1p may function together.

We next asked whether yeast passenger protein homologues also have a role in regulating septins. To address this question, we used preexisting alleles of *IPL1* or *SLI15* and created conditional alleles of *BIR1*. Mutant strains were engineered to express Cdc11-GFP, and septins were analyzed after growth of cells at permissive (25°C) or nonpermissive (37°C) temperatures. At 25°C, all strains exhibited normally organized septins; when shifted to 37°C, *ipl1-321*, *ipl1-182*, *sli15-3*, and *sli15-1* all exhibited only a small increase in cells with disorganized septins, equivalent to isogenic wild-type control strains (Fig. 8, A and B; and Fig. S4). In contrast, growth of *bir1-33* at nonpermissive temperature gave rise to a dramatic defect in septin organization in a significant percentage of cells. Septins appeared in disorganized clusters and rings were often fragmented or partially formed (Fig. 8 B and Fig. S4). This phenotype is remarkably similar to that observed for mutants in CBF3 assembly and shows that Bir1p and CBF3 function together to regulate septins.

To further assess the role of passenger protein homologues in regulating septins, we analyzed the ability of *IPL1*, *SLI15*, and *BIR1* mutants to form an axial bud pattern during cell division. Mutant strains and their isogenic wild-type controls were grown at permissive (25°C), semipermissive (34°C), and nonpermissive (37°C) temperatures to compromise gene function, but still allow cells to complete multiple rounds of budding; bud scars were visualized with Calcofluor white (Fig. 8 D). The wild-type W303a control for the *IPL1* mutant exhibited a high degree of nonaxial budding at all temperatures. However, *ipl1-321* showed a slight decrease in nonaxial budding, suggesting that compromising Ipl1p function does not exacerbate cell polarity defects in this strain background. Consistent with this conclusion, a mutant allele of the Ipl1p binding partner *sli15-3* exhibited low levels of nonaxial budding at all temperatures, comparable to the isogenic wild-type control. Similar results were obtained using the *sli15-1* and *ipl1-182* alleles, showing that multiple alleles that affect the kinase complex are normal for septin function (Fig. S4). In contrast, *bir1-33* exhibited a dramatic increase in nonaxial bud growth at semipermissive and nonpermissive temperatures compared with the isogenic wild-type control (from 10 to 85%; Fig. 8, D and E). Although we cannot exclude the possibility that the *IPL1* and *SLI15* alleles we analyzed remain partially functional, our data is consistent with Bir1p having a distinct role in regulating septins (see Discussion). Together, these data provide strong evidence that CBF3 and Bir1p form a passenger complex to regulate septins and cytokinesis.

Discussion

To maintain mitotic fidelity, it is necessary to coordinate the onset of cytokinesis with the completion of chromosome segregation in anaphase. In metazoans, it has been suggested that passenger proteins link these events by transferring from kinetochores to the central spindle in anaphase (Vagnarelli and Earnshaw, 2004). However, neither the events that regulate the transfer of passenger proteins nor how passenger proteins control cytokinesis are understood. This work provides evidence that the kinetochore scaffold complex CBF3 has a second novel role in regulating cytokinesis that is independent of its well studied function in chromosome segregation. We conclude that, like animal cells, the simple eukaryote *S. cerevisiae* uses passenger proteins to regulate cytokinesis and that septins are a novel target of a Bir1p-passenger complex.

Although it is surprising that a sequence-specific DNA-binding complex has a second role in regulating septins, our previous work suggested that the dynamic assembly and turnover

of CBF3 continues after kinetochores have formed on CEN DNA; this observation led us to propose that the CBF3 cycle may be required for processes independent of its role in nucleating kinetochores and segregating chromosomes (Rodrigo-Brenni et al., 2004). We show that septin organization is sensitive to perturbations in the CBF3 cycle; mutations that inhibit CBF3 assembly (*skp1-4*) or the depletion of CBF3 from cells (raffinose/*GAL1-CTF13*) cause defects in the organization of septins. Blocking the turnover of CBF3 complexes (*skp1-3*) or overproducing CBF3 (galactose/*GAL1-CTF13*) also compromises septin organization. Although the precise role of the CBF3 cycle in regulating septins remains unclear, we speculate that this cycle may maintain the balance of CBF3 complexes that form kinetochore scaffolds versus passenger complexes.

The role of septins in cytokinesis has been well established in multiple systems (Kinoshita, 2003; Longtine and Bi, 2003). Recently, in yeast, septins have been shown to form a diffusion barrier in anaphase, which helps to ensure the proper localization of the membrane and cell wall synthesis machinery required for cytokinesis (Dobbelaere and Barral, 2004). Even subtle perturbations in septin organization lead to diffusion of these proteins away from the mother bud neck and failed cytokinesis (Dobbelaere and Barral, 2004). Certainly, the septin defects that we observed are consistent with the loss of this diffusion barrier activity and may explain the defect in cytokinesis. Septin organization may be compromised because of a failure to properly regulate their dynamics late in anaphase. Although our data support a role for CBF3 in regulating these dynamics, we imagine that this role is indirect, possibly through changes in posttranslational modifications of septins. The regulation of septin dynamics during the anaphase–G1 transition has been linked to protein phosphorylation, as well as SUMO (small ubiquitin-like modifier) modification (Johnson and Blobel, 1999; Johnson and Gupta, 2001; Shih et al., 2002; Kinoshita, 2003; Martin and Konopka, 2004). It has been reported that the PP2A subunit Rts1p localizes to kinetochores in metaphase and to septins in anaphase (Dobbelaere et al., 2003). Our comparison of the septin phenotypes suggests that *RTS1* and CBF3 differentially regulate septins. Nonetheless, it is striking that defects in all of these pathways (i.e., phosphorylation, sumoylation, and ubiquitination) affect aspects of septin stability. It is possible that CBF3 intersects with multiple regulatory pathways that control septin separation and disassembly, thus creating a more complex phenotype than individual mutations in septin regulators.

Our observation that inhibiting CBF3 assembly also affects septins in α factor–treated cells has multiple implications. We conclude that CBF3 assembly is required in G1 to regulate septins, completely independent of its role in segregating chromosomes. The belief that septins are probably dynamic as they reform from a shmoo into bud configurations supports a role for CBF3 in regulating septin dynamics. Therefore, this result may reflect a requirement of CBF3 to regulate septins when they are at their most dynamic; e.g., during the disassembly of septin rings at the anaphase–G1 transition. Furthermore, this finding may reflect a similar need in anaphase and mating cells to coordinate microtubules, actin, and nuclear movement. In this light, it is intriguing to note that, during mating in yeast microtubules, nucleate from opposite spindle poles must find each other with the help of the septin–actin cortex and form a plus-end–plus-end interaction. This orientation of microtubules is analogous to the orientation of anaphase interpolar microtubules and suggests that these two structures may share some of the same machinery to form their attachment sites.

The direct interaction between CBF3 and Bir1p indicates that these proteins function together to regulate septins in anaphase. The importance of this interaction, and the possibility that other proteins participate in regulating septins, remains to be explored. Our genetic data suggest that neither Sli15p nor Ipl1p are involved in regulating septins (Fig. 7). This is somewhat surprising, as Survivin, which is the Bir1p homologue in animal cells, interacts with Aurora B (Ipl1p) and INCENP (Sli15p), and Bir1p has been observed to copurify with Sli15p from yeast (Wheatley et al., 2001; Cheeseman et al., 2002). Although we have examined multiple alleles of *IPL1* and *SLI15*, it is possible that these mutations remain functional for regulating septins. *ipl1-321* has been reported to eliminate the kinase activity of Ipl1p (Biggins et al., 1999), showing that the kinase function of Ipl1p is not critical for septin regulation. Caution is also required when interpreting the alleles of *SLI15*, especially because *sli15-1* was isolated as a synthetic lethal mutant of *ipl1-2* and *sli15-3* displays a very similar phenotype to *ipl1-2* (Kim et al., 1999); it may well be that these *SLI15* alleles are specifically defective in regulating Ipl1p kinase activity but wild type for its role in a putative septin-regulating complex. Nonetheless, the high frequency of conditional *BIR1* alleles that we have isolated with septin defects demonstrates that Bir1p has a role distinct from the Sli15p–Ipl1p complex in regulating septin dynamics (unpublished data). Studies in animal cells are consistent with this possibility, as Survivin is found in multiple passenger-type complexes (Gassmann et al., 2004). Whether the CBF3–Bir1p passenger complex directly interacts with septins or whether it influences intermediates to regulate septins is an important question that remains to be addressed.

Are these novel findings regarding passenger complexes in yeast relevant to higher eukaryotes? Although CBF3 is not conserved, there are some very intriguing similarities between budding yeast and higher eukaryote passenger complexes. For example, Sgt1p, Skp1p, and HSP90 regulate the CBF3 cycle and are highly conserved (Kitagawa et al., 1999; Bansal et al., 2004; Lingelbach and Kaplan, 2004; Rodrigo-Brenni et al., 2004). It has been shown that the small interfering RNA of human *SGT1* compromises kinetochore formation, supporting the argument that a similar cycle may impact human kinetochores (Steensgaard et al., 2004). In higher eukaryotes, CENP-A–containing nucleosomes are critical for kinetochore formation. Like CBF3, CENP-A is a target of the Aurora B passenger kinase (Ipl1p in yeast; Sassoon et al., 1999; Zeitlin et al., 2001). Remarkably, overexpression of a CENP-A mutant that prevents Aurora B phosphorylation results in cytokinetic defects (Zeitlin et al., 2001). These results raise the interesting possibility that kinetochore scaffold complexes share a conserved

dual role in nucleating kinetochores and in coordinating chromosome segregation with cytokinesis. Finally, the transfer of Bir1p from kinetochores to interpolar microtubules and its requirement for cytokinesis is reminiscent of Survivin, its counterpart in mammalian cells (Yang et al., 2004). Therefore, we conclude that an analogous system of proteins function in yeast to coordinate chromosome segregation with septin organization and cytokinesis.

Materials and methods

Yeast growth and strain construction

Yeast strains were grown in standard yeast extract/bactopeptone (YEP) media supplemented with 2% dextrose or raffinose and 2% galactose, as indicated (Guthrie and Fink, 1991). A plasmid shuffle strategy was used to introduce *BIR1* mutants into a haploid strain containing a chromosomal deletion of *BIR1* and an episomal copy of *BIR1* marked with *URA3* (see Table I for more information). Error-prone PCR was used to introduce random mutations into the *BIR1*-coding region. Mutants were introduced into yeast by cotransfection of *BIR1* PCR product and a gapped plasmid, as previously described (Umen and Guthrie, 1995). The wild-type copy of *BIR1* was selected against 5-fluorotic acid and temperature-sensitive alleles were isolated. Other mutant alleles were grown as indicated in the figure legends.

For yeast two-hybrid analyses, *NDC10* and *SLI15* were cloned into the *GAL4*-binding domain vector (James et al., 1996) using SmaI and SalI (pUD256) and BamHI and PstI (pUD364) restriction sites, respectively. The DNA-encoding amino acids 550–954 of Bir1p or the entire coding region of Ipl1p and Sli15p were cloned into the *GAL4* activation domain vector with BamHI and SalI (pUD257), BamHI and SalI (pUD403), and BamHI and PstI (pUD428), respectively (James et al., 1996). Transformants were isolated on minimal media plates lacking tryptophan and leucine and assayed on plates lacking histidine to detect two-hybrid interactions.

Table I. **Strains used in this study**

Strain	Genotype	Reference
906	*MATa ade2-1 his3-11 leu2-3,112 ura3-1 trp1-1 can1-100 dam1Δ::HIS3 dam1-1::URA3*	Jones et al., 2001
CCY482-13D	*MATa ade2 leu2-3,112 ura3-52 his3-Δ200 lys2::HIS3:lys2*	Kim et al., 1999
CCY82-13D-1-1	*MATa ade2 leu2-3,112 ura3-52 his3-Δ200 lys2::HIS3:lys2 sli15-3*	Kim et al., 1999
CJY231	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 spc25Δ::HIS3MX4 LEU2Δ1::spc25-7*	Janke et al., 2001
cse4-1	*MATα ade2-101 his3-11,15 trp1Δ901 ura3 leu2-3 cse4-1*	Stoler et al., 1995
DJ553	*MATa ade2-101 his3-Δ200 leu2 lys2-801 trp1-Δ63 ura3-52 RTS1-GFP::TRP1*	Dobbelaere et al., 2003
DJ723	*MATa ade2-101 his3-Δ200 leu2 lys2-801 trp1-Δ63 ura3-52 RTS1Δ::KANmX6*	Dobbelaere et al., 2003
KSC410	*MATa ura3-1 ade2-1 trp1-1 his3-11 leu2-3,112 can1-100*	This study
KSC885	*MATα sgt1Δ::HIS3 sgt1-107::LEU2*	This study
KSC988	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-TRP1::CTF13 SWE1Δ::TRP1*	This study
KSC1028	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-TRP1::CTF13*	Rodrigo-Brenni et al., 2004
KSC1234	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GFP-BIR1::HIS3*	This study
KSC1279	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GAL1-3XHA-TRP1::CTF13 CDC11::GFP HIS3*	This study
KSC1280	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIIIhis3sup11 GFP-CDC11::TRP1*	This study
KSC1660	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	This study
KSC1789	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 GFP-CDC11::TRP1*	This study
KSC2142	*MATa ura3-1 ade2-1 trp1-1 his3-11 leu2-3,112 can1-100 GFP-CDC11::TRP1*	This study
KSC2144	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 LSY2 BAR1 can1-100 ade2-1 ipl1-321 GFP-CDC11::TRP1*	This study
KSC2164	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 BIR1Δ::HIS3 GFP-CDC11::TRP1 bir1-33::LEU2*	This study
KSC2166	*MATa ade2 leu2-3,112 ura3-52 his3-Δ200 lys2::HIS3:lys2 sli15-3 GFP-CDC11::KAN*	This study
KSC2167	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3- 200 leu2-Δ1 BIR1Δ::HIS3 GFP-CDC11::TRP1 bir1-30::LEU2*	This study
ndc80-1	*MATa leu2-3,112 ura3 ade2-1 his3-11,15 trp1-1 can1-100 ndc80-1::ndc80*	Wigge et al., 1998
RJD667	*leu2 trp1 ura3 pep4::TRP1 cdc34-2*	Deshaies and Kirschner, 1995
SBY321	*MATα ura3-1 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ lys2Δ:pGAL-Δ176-CLB2:LYS2 ipl1-321*	Biggins et al., 1999
SBY630	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 LSY2 BAR1 can1-100 ade2-1 ipl1-321*	Gift from Sue Biggins
JK418	*MATa ura3-1 ade2-1 trp1-1 his3-11 leu2-3,112 can1-100, ndc10-1*	Goh and Kilmartin, 1993
YKH8	*MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 ctf19-58*	Basrai et al., 1996
YKH10	*MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 ctf19-26*	Basrai et al., 1996
YKK57	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 sgt1-5::LEU2*	Kitagawa et al., 1999
YKK66	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 sgt1-3::LEU2*	Kitagawa et al., 1999
YPH500	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1his3-Δ200 leu2-Δ1*	Sikorski and Hieter, 1989
YPH1015	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIII (CEN3.L.YPH983) HIS3 SUP11*	Connelly and Hieter, 1996
YPH1161	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ1::TRP1 skp1-4::LEU2 CFIII (CEN3.L.YPH983) HIS3 SUP11*	Connelly and Hieter, 1996
YPH1172	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 skp1Δ1::TRP1 skp1-3::LEU2 CFIII (CEN3.L.YPH983) HIS3 SUP11*	Connelly and Hieter, 1996
XH230	*MATa ade2-101 his3-1 leu2-3,112 ura3-11 trp1-1 can1-100 mtw1-1::TRP1*	Goshima and Yanagida, 2000

In vitro binding assay

Recombinant GST and GST fused to Bir1p were produced in insect cells using the Fastbac Baculovirus expression system (Invitrogen). Proteins were isolated after cell lysis, as previously described (Rodrigo-Brenni et al., 2004). Ndc10p was translated in vitro using the TNT T7 Quick Coupled Translation/Transcription System (Promega). 0.5 μg of template DNA and 20 μCi of ^{35}S-methionine were used in the translation reaction, as per the manufacturer's recommendations. Glutathione–Sepharose (GE Healthcare) was used to isolate 1 μg GST-Bir1p or GST and mixed with half of the translation reaction in 250 μL of binding buffer (50 mM Tris, pH 7.0, 50 mM KCl, 0.05% Triton X-100, 1 mM DTT, and 10% glycerol). Samples were rocked at 4°C for 3 h, the resin was washed three times with binding buffer, and proteins were resolved using SDS-PAGE and analyzed using Phosphorimager analysis (GE Healthcare).

Cell cycle point of execution

In these experiments, strains contained either the wild-type *CTF13* or the *GAL1* promoter integrated upstream of the *CTF13* coding sequences and fused to a triple HA epitope tag, as previously reported (Rodrigo-Brenni et al., 2004). Cells were grown at 30°C to log phase in YEP media plus 2% galactose and 2% raffinose. The medium was adjusted to 0.1 M hydroxyurea, and >90% of cells arrested with large buds over a 3-h period. Cells were centrifuged and resuspended in hydroxyurea containing YEP with 2% raffinose or 2% raffinose/galactose, as indicated in the figure legends, and incubated at 34°C for 2 h. Cells were released from the block by centrifugation and washing with fresh medium containing the carbon source indicated in the figure legends and were allowed to proceed through anaphase or arrested during metaphase with 15 μg/ml nocodazole. Cells were collected after 90 min and analyzed by fluorescent microscopy. Abnormal septins were scored based on Cdc11-GFP distribution; all observations were confirmed using immunofluorescence with anti-Cdc11 antibodies.

Cells were similarly arrested using 10 μg/ml α factor (American Peptide, Inc.) in medium containing 2% raffinose/galactose. Cells were centrifuged and washed into fresh medium containing α factor and 2% raffinose or 2% raffinose/galactose for 2 h. Finally, cells were centrifuged and washed into fresh medium containing the carbon source indicated in the figure legends and 0.1 M hydroxyurea. Samples were collected after 120 min and analyzed by fluorescent microscopy.

Fluorescent and light microscopy

Cells containing GFP gene fusions were collected at the indicated time points in the figure legends and placed on agarose pads containing a carbon source identical to that of the culture media, as described previously (Hoepfner et al., 2000; Rodrigo-Brenni et al., 2004). For fluorescent staining, cells were grown as indicated in the figure legends, incubated in 3.7% formaldehyde for 1 h at the culture temperature, and processed for DAPI, tubulin, and Cdc11p staining (tubulin or Cdc11p antibodies were diluted 1:400 in PBS, 0.2% gelatin, and 0.02% NaN3) as described previously (Guthrie and Fink, 1991; Rodrigo-Brenni et al., 2004). Phalloidin staining was performed by resuspending the cells in 0.1 M Tris/HCl, pH 9.4, and 10 mM DTT and incubating at 25°C for 8 min. Cells were washed twice with ice-cold PBS; 1.0×10^7 cells were resuspended in 100 μL PBS and supplemented with 4.0×10^{-3} units of Texas red–conjugated phalloidin (Sigma Aldrich) and 2.5 μg DAPI. Cells were incubated in the dark for 1.5 h, washed twice in PBS, resuspended in 20 μL PBS, and applied to poly-L-lysine–coated coverslips. To visualize bud scars, cells were resuspended in PBS with 20 μg/ml Calcofluor white (Fluorescent Brightener 28; Sigma Aldrich), incubated at 25°C for 5 min, washed in PBS, and visualized as described above for GFP fusions.

Fluorescent images were collected using an epifluorescence microscope (model E600; Nikon) equipped with either 60× (NA 1.35) or 100× (NA 1.4) oil immersion lenses (Nikon) and recorded with a charge-coupled device camera (model Orca ER; Hamamatsu) controlled by Simple PCI software (Compix Inc.). Color was added to images using Adobe Photoshop version 7.0. The rat tubulin antibody was purchased from Sigma-Aldrich. The polyclonal antibody against Cdc11p was provided by D. Kellogg (University of California, Santa Cruz, Santa Cruz, CA; Carroll et al., 1998).

Online supplemental material

Fig. S1 shows changes in the kinetics of disorganized septins and abnormal bud morphologies after inhibition of CBF3 complexes. Fig. S2 depicts examples of septin organization in strains containing mutations in inner or outer kinetochore genes. Fig. S3 provides an analysis of septin organization in wild-type and mutant strains of *RTS1* and suggests that *RTS1* and CBF3 are in parallel pathways with respect to septin regulation. Fig. S4 presents septin organization in additional *bir1*, *sli15*, and *ipl1* conditional alleles. Video 1 shows the behavior of Cdc11-GFP in anaphase of wild-type cells. Video 2 shows the behavior of Cdc11-GFP in an anaphase inhibited for CBF3 assembly. Video 3 shows an example of septin behavior in an unbudded cell inhibited for CBF3 assembly. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200507017/DC1.

We would like to thank Doug Kellogg, Kerry Bloom, and John Pringle for helpful discussions; we also thank Doug Kellogg for providing antibodies to Cdc11p.

We acknowledge the support of a grant from the American Cancer Society (RSG-02-035-01-CCG) to K.B. Kaplan.

Submitted: 6 July 2005
Accepted: 24 October 2005

References

Bansal, P.K., R. Abdulle, and K. Kitagawa. 2004. Sgt1 associates with Hsp90: an initial step of assembly of the core kinetochore complex. *Mol. Cell. Biol.* 24:8069–8079.

Basrai, M.A., J. Kingsbury, D. Koshland, F. Spencer, and P. Hieter. 1996. Faithful chromosome transmission requires Spt4p, a putative regulator of chromatin structure in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 16: 2838–2847.

Biggins, S., F.F. Severin, N. Bhalla, I. Sassoon, A.A. Hyman, and A.W. Murray. 1999. The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes Dev.* 13:532–544.

Bouck, D.C., and K.S. Bloom. 2005. The kinetochore protein Ndc10p is required for spindle stability and cytokinesis in yeast. *Proc. Natl. Acad. Sci. USA.* 102:5408–5413.

Buvelot, S., S.Y. Tatsutani, D. Vermaak, and S. Biggins. 2003. The budding yeast Ipl1/Aurora protein kinase regulates mitotic spindle disassembly. *J. Cell Biol.* 160:329–339.

Carroll, C.W., R. Altman, D. Schieltz, J.R. Yates, and D. Kellogg. 1998. The septins are required for the mitosis-specific activation of the Gin4 kinase. *J. Cell Biol.* 143:709–717.

Cheeseman, I.M., S. Anderson, M. Jwa, E.M. Green, J. Kang, J.R. Yates III, C.S. Chan, D.G. Drubin, and G. Barnes. 2002. Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell.* 111:163–172.

Cleveland, D.W., Y. Mao, and K.F. Sullivan. 2003. Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell.* 112:407–421.

Connelly, C., and P. Hieter. 1996. Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. *Cell.* 86:275–285.

Deshaies, R.J., and M. Kirschner. 1995. G1 cyclin-dependent activation of p34-cdc28 (Cdc28p) in vitro. *Proc. Natl. Acad. Sci. USA.* 92:1181–1186.

Dobbelaere, J., and Y. Barral. 2004. Spatial coordination of cytokinetic events by compartmentalization of the cell cortex. *Science.* 305:393–396.

Dobbelaere, J., M.S. Gentry, R.L. Hallberg, and Y. Barral. 2003. Phosphorylation-dependent regulation of septin dynamics during the cell cycle. *Dev. Cell.* 4:345–357.

Ducat, D., and Y. Zheng. 2004. Aurora kinases in spindle assembly and chromosome segregation. *Exp. Cell Res.* 301:60–67.

Espelin, C.W., K.B. Kaplan, and P.K. Sorger. 1997. Probing the architecture of a simple kinetochore using DNA–protein crosslinking. *J. Cell Biol.* 139:1383–1396.

Flescher, E.G., K. Madden, and M. Snyder. 1993. Components required for cytokinesis are important for bud site selection in yeast. *J. Cell Biol.* 122:373–386.

Ford, S.K., and J.R. Pringle. 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the CDC11 gene product and the timing of events at the budding site. *Dev. Genet.* 12:281–292.

Gardner, R.D., A. Poddar, C. Yellman, P.A. Tavormina, M.C. Monteagudo, and D.J. Burke. 2001. The spindle checkpoint of the yeast *Saccharomyces cerevisiae* requires kinetochore function and maps to the CBF3 domain. *Genetics.* 157:1493–1502.

Gassmann, R., A. Carvalho, A.J. Henzing, S. Ruchaud, D.F. Hudson, R. Honda, E.A. Nigg, D.L. Gerloff, and W.C. Earnshaw. 2004. Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. *J. Cell Biol.* 166:179–191.

Goh, P.Y., and J.V. Kilmartin. 1993. NDC10: a gene involved in chromosome segregation in *Saccharomyces cerevisiae*. *J. Cell Biol.* 121:503–512.

Goshima, G., and M. Yanagida. 2000. Establishing biorientation occurs with precocious separation of the sister kinetochores, but not the arms, in the early spindle of budding yeast. *Cell*. 100:619–633.

Guthrie, C., and G.R. Fink, editors. 1991. *Guide to Yeast Genetics and Molecular Biology.* Methods in Enzymology, vol. 194. San Diego: Academic Press.

Hoepfner, D., A. Brachat, and P. Philippsen. 2000. Time-lapse video microscopy analysis reveals astral microtubule detachment in the yeast spindle pole mutant cnm67. *Mol. Biol. Cell.* 11:1197–1211.

Hyland, K.M., J. Kingsbury, D. Koshland, and P. Hieter. 1999. Ctf19p: a novel kinetochore protein in *Saccharomyces cerevisiae* and a potential link between the kinetochore and mitotic spindle. *J. Cell Biol.* 145:15–28.

James, P., J. Halladay, and E.A. Craig. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics.* 144:1425–1436.

Janke, C., J. Ortiz, J. Lechner, A. Shevchenko, M.M. Magiera, C. Schramm, and E. Schiebel. 2001. The budding yeast proteins Spc24p and Spc25p interact with Ndc80p and Nuf2p at the kinetochore and are important for kinetochore clustering and checkpoint control. *EMBO J.* 20:777–791.

Johnson, E.S., and G. Blobel. 1999. Cell cycle–regulated attachment of the ubiquitin-related protein SUMO to the yeast septins. *J. Cell Biol.* 147:981–994.

Johnson, E.S., and A.A. Gupta. 2001. An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell.* 106:735–744.

Jones, M.H., X. He, T.H. Giddings, and M. Winey. 2001. Yeast Dam1p has a role at the kinetochore in assembly of the mitotic spindle. *Proc. Natl. Acad. Sci. USA.* 98:13675–13680.

Kaitna, S., M. Mendoza, V. Jantsch-Plunger, and M. Glotzer. 2000. Incenp and an aurora-like kinase form a complex essential for chromosome segregation and efficient completion of cytokinesis. *Curr. Biol.* 10:1172–1181.

Kaplan, K.B., and P.K. Sorger. 1997. Purification of sequence-specific DNA-binding proteins. *In* Protein Function: A Practical Approach. T.E. Creighton, editor. Oxford University Press, Oxford. 245–278.

Kaplan, K.B., A.A. Hyman, and P.K. Sorger. 1997. Regulating the yeast kinetochore by ubiquitin-dependent degradation and Skp1p-mediated phosphorylation. *Cell.* 91:491–500.

Kim, H.B., B.K. Haarer, and J.R. Pringle. 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the *CDC3* gene product and the timing of events at the budding site. *J. Cell Biol.* 112:535–544.

Kim, J.H., J.S. Kang, and C.S. Chan. 1999. Sli15 associates with the ipl1 protein kinase to promote proper chromosome segregation in *Saccharomyces cerevisiae*. *J. Cell Biol.* 145:1381–1394.

Kinoshita, M. 2003. Assembly of mammalian septins. *J. Biochem. (Tokyo).* 134:491–496.

Kitagawa, K., D. Skowyra, S.J. Elledge, J.W. Harper, and P. Hieter. 1999. SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. *Mol. Cell.* 4:21–33.

Lampson, M.A., K. Renduchitala, A. Khodjakov, and T.M. Kapoor. 2004. Correcting improper chromosome-spindle attachments during cell division. *Nat. Cell Biol.* 6:232–237.

Lew, D.J., and D.J. Burke. 2003. The spindle assembly and spindle position checkpoints. *Annu. Rev. Genet.* 37:251–282.

Lingelbach, L.B., and K.B. Kaplan. 2004. The interaction between Sgt1p and Skp1p is regulated by HSP90 chaperones and is required for proper CBF3 assembly. *Mol. Cell. Biol.* 24:8938–8950.

Longtine, M.S., and E. Bi. 2003. Regulation of septin organization and function in yeast. *Trends Cell Biol.* 13:403–409.

Longtine, M.S., H. Fares, and J.R. Pringle. 1998. Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. *J. Cell Biol.* 143:719–736.

Martin, S.W., and J.B. Konopka. 2004. SUMO modification of septin-interacting proteins in *Candida albicans*. *J. Biol. Chem.* 279:40861–40867.

McAinsh, A.D., J.D. Tytell, and P.K. Sorger. 2003. Structure, function, and regulation of budding yeast kinetochores. *Annu. Rev. Cell Dev. Biol.* 19:519–539.

Mortensen, E.M., H. McDonald, J. Yates III, and D.R. Kellogg. 2002. Cell cycle-dependent assembly of a Gin4-septin complex. *Mol. Biol. Cell.* 13:2091–2105.

Murata-Hori, M., M. Tatsuka, and Y.L. Wang. 2002. Probing the dynamics and functions of aurora B kinase in living cells during mitosis and cytokinesis. *Mol. Biol. Cell.* 13:1099–1108.

Pereira, G., and E. Schiebel. 2003. Separase regulates INCENP-Aurora B anaphase spindle function through Cdc14. *Science.* 302:2120–2124.

Petersen, J., J. Paris, M. Willer, M. Philippe, and I.M. Hagan. 2001. The *S. pombe* aurora-related kinase Ark1 associates with mitotic structures in a stage dependent manner and is required for chromosome segregation. *J. Cell Sci.* 114:4371–4384.

Richman, T.J., M.M. Sawyer, and D.I. Johnson. 1999. The Cdc42p GTPase is involved in a G2/M morphogenetic checkpoint regulating the apical-isotropic switch and nuclear division in yeast. *J. Biol. Chem.* 274:16861–16870.

Rodrigo-Brenni, M.C., S. Thomas, D.C. Bouck, and K.B. Kaplan. 2004. Sgt1p and Skp1p modulate the assembly and turnover of CBF3 complexes required for proper kinetochore function. *Mol. Biol. Cell.* 15:3366–3378.

Russell, I.D., A.S. Grancell, and P.K. Sorger. 1999. The unstable F-box protein p58-Ctf13 forms the structural core of the CBF3 kinetochore complex. *J. Cell Biol.* 145:933–950.

Sassoon, I., F.F. Severin, P.D. Andrews, M.R. Taba, K.B. Kaplan, A.J. Ashford, M.J. Stark, P.K. Sorger, and A.A. Hyman. 1999. Regulation of *Saccharomyces cerevisiae* kinetochores by the type 1 phosphatase Glc7p. *Genes Dev.* 13:545–555.

Shih, H.P., K.G. Hales, J.R. Pringle, and M. Peifer. 2002. Identification of septin-interacting proteins and characterization of the Smt3/SUMO-conjugation system in *Drosophila*. *J. Cell Sci.* 115:1259–1271.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 122:19–27.

Sreenivasan, A., and D. Kellogg. 1999. The elm1 kinase functions in a mitotic signaling network in budding yeast. *Mol. Cell. Biol.* 19:7983–7994.

Steensgaard, P., M. Garre, I. Muradore, P. Transidico, E.A. Nigg, K. Kitagawa, W.C. Earnshaw, M. Faretta, and A. Musacchio. 2004. Sgt1 is required for human kinetochore assembly. *EMBO Rep.* 5:626–631.

Stemmann, O., A. Neidig, T. Kocher, M. Wilm, and J. Lechner. 2002. Hsp90 enables Ctf13p/Skp1p to nucleate the budding yeast kinetochore. *Proc. Natl. Acad. Sci. USA*. 99:8585–8590.

Stoler, S., K.C. Keith, K.E. Curnick, and M. Fitzgerald-Hayes. 1995. A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. *Genes Dev*. 9:573–586.

Tanaka, T.U., N. Rachidi, C. Janke, G. Pereira, M. Galova, E. Schiebel, M.J. Stark, and K. Nasmyth. 2002. Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell.* 108:317–329.

Terada, Y. 2001. Role of chromosomal passenger complex in chromosome segregation and cytokinesis. *Cell Struct. Funct.* 26:653–657.

Terada, Y., M. Tatsuka, F. Suzuki, Y. Yasuda, S. Fujita, and M. Otsu. 1998. AIM-1: a mammalian midbody-associated protein required for cytokinesis. *EMBO J.* 17:667–676.

Umen, J.G., and C. Guthrie. 1995. A novel role for a U5 snRNP protein in 3′ splice selection. *Genes Dev.* 9:855–868.

Vagnarelli, P., and W.C. Earnshaw. 2004. Chromosomal passengers: the four-dimensional regulation of mitotic events. *Chromosoma.* 113:211–222.

Wheatley, S.P., A. Carvalho, P. Vagnarelli, and W.C. Earnshaw. 2001. INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis. *Curr. Biol.* 11:886–890.

Wigge, P.A., and J.V. Kilmartin. 2001. The Ndc80p complex from *Saccharomyces cerevisiae* contains conserved centromere components and has a function in chromosome segregation. *J. Cell Biol.* 152:349–360.

Wigge, P.A., O.N. Jensen, S. Holmes, S. Soues, M. Mann, and J.V. Kilmartin. 1998. Analysis of the *Saccharomyces* spindle pole by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. *J. Cell Biol.* 141: 967–977.

Yang, D., A. Welm, and J.M. Bishop. 2004. Cell division and cell survival in the absence of survivin. *Proc. Natl. Acad. Sci. USA.* 101:15100–15105.

Yoon, H.J., and J. Carbon. 1999. Participation of Bir1p, a member of the inhibitor of apoptosis family, in yeast chromosome segregation events. *Proc. Natl. Acad. Sci. USA*. 96:13208–13213.

Zeitlin, S.G., R.D. Shelby, and K.F. Sullivan. 2001. CENP-A is phosphorylated by Aurora B kinase and plays an unexpected role in completion of cytokinesis. *J. Cell Biol.* 155:1147–1157.