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

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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, Sgf1p, 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).
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 inter-polar 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 pheromones 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 (t1/2 = 10 min) and CBF3 complexes (t1/2 = 25 min) as measured in cell extracts (Rodriguo-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 (Rodriguo-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...
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; 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).
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/H11005 32 min; GAL1-CTF13/H11005 48 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 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 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 re-
leased 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 re-
leased from the α factor arrest and CBF3 assembly was inhib-
ited, 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 (Rod-
orio-Brenni et al., 2004), it is possible that there are subtle
changes in outer kinetochore complexes that affect septin orga-
nization. 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 Kil-
Martin, 2001), making it unlikely that a pre-anaphase arrest
masks a septin defect. In contrast, alleles that affect CBF3 as-
sembly exhibit a high percentage of cells with disorganized sep-
tins (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 misori-
ented 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 pre-
vents 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), re-
results in an even higher percentage of cells with disorganized
septins. SKP1 and SGT1 have also been implicated in the func-
tion of the Skp1-cullin-F-box (SCF)–E3 ubiquitin ligase com-
plex. 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

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 inap-
propriately 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 simi-
lar to that recently shown by Bouck and Bloom (2005). How-
ever, 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 sep-
tin 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 4. Septin organization in shmooos 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 indi-
cate cells with disorganized septins. (C) The total percentage of cells with
abnormal septins was calculated. Bar, 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.
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/H9004 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 RTS1Δ was deleted (18%); we never observed this phenotype in 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 RTS1Δ strain and isolated several meiotic products containing both mutations. Even when grown on galactose, the double mutants (GAL1-CTF13 and RTS1Δ) 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 Bir1p (Survivin), Ipl1p (Aurora B) and Sl15p (INCENP) encode likely homologues of known passenger proteins, and both Ipl1p and Sl15p 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 Bir1p 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 6](https://www.jcb.org/cgi/content/full/jcb.200507017/DC1)

**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.

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**Figure 7.** Bir1p localizes to interpolar microtubules and interacts with Ndc10p. (A) Bir1-GFP was imaged in prometaphase 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 35S-methionine. The translated protein was incubated with GST-Bir1p or GST and the fraction of the Ndc10p bound to the beads was calculated.

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**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.

![Figure 7](https://www.jcb.org/cgi/content/full/jcb.200507017/DC1)

**Figure 7.** Bir1p localizes to interpolar microtubules and interacts with Ndc10p. (A) Bir1-GFP was imaged in prometaphase 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 35S-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-pasenger 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 (Dobbelare and Barral, 2004). Even subtle perturbations in septin organization lead to diffusion of these proteins away from the mother bud neck and failed cytokinesis (Dobbelare 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 yeast relevant to higher eukaryotes? Although CBF3 is not a synthetic lethal mutant of ipi1-2 and sli15-3 displays a very similar phenotype to ipi1-2 (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., 2002; Lingelbach and Kaplan, 2004; Rodrigo-Brenni et al., 2004). It has been shown that the small interfering RNA of human SGTI 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
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-fluorotricin 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 Sall (pUD256) and BamH I and PstI (pUD364) restriction sites, respectively. The DNA-encoding amino acids 550–954 of Bir1 or the entire coding regions of Ipl1p and Sli15p were cloned into the GAL4 activation domain vector with BamH I and Sall (pUD257), BamH I and Sall (pUD403), and BamH I 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                     | Jones et al., 2001                 |
| CCY482-13D      | MATa ade2 leu2-3,112 ura3-52 his3-2000 lys2-2:His3 lys2                   | Kim et al., 1999                   |
| CCY82-13D-1-1   | MATa ade2 leu2-3,112 ura3-52 his3-2000 lys2-2:His3 lys2                   | Kim et al., 1999                   |
| CJIY231         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 spc25::LEU2::::spc27 | Janke et al., 2001                 |
| cse4-1          | MATa ade2-101 his3-11,15 trp1-1::63 ura3 leu2-3 cse4-1                   | Stoler et al., 1995                |
| DJ553           | MATa ade2-101 his3-2000 leu2-801 trp1-1::63 ura3-52 RtsI::GFP::TRP1       | Dobbleare et al., 2003             |
| DJ723           | MATa ade2-101 his3-2000 leu2-801 trp1-1::63 ura3-52 RtsI::KANmX6          | Dobbleare et al., 2003             |
| KSC410          | MATa ade2-101 trp1-1::63 his3-11 leu2-3,112 can1-100                     | This study                         |
| KSC885          | MATa sgt1-1::His3 sgt1-107::::EU2                                        | This study                         |
| KSC988          | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801 GAL1-3XHA-TRP1::CTF13 | This study                         |
| KSC1028         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801 GAL1-3XHA-TRP1::CTF13 | Rodrigo-Brenni et al., 2004        |
| KSC1234         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801 GFP::BIR1::His3 | This study                         |
| KSC1279         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801 GAL1-3XHA-TRP1::CTF13 | This study                         |
| KSC1280         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801 GFP::CDC11::::GFP | This study                         |
| KSC1660         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801              | This study                         |
| KSC1789         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801 GFP::CDC11::::TRP1 | This study                         |
| KSC2142         | MATa ura3-1 ade2-1 trp1-1::63 his3-11 leu2-3,112 can1-100::GFP::CDC11::::TRP1 | This study                         |
| KSC2144         | MATa ura3-1 leu2-3,112 his3-11 trp1-1::63 his3-11 leu2-3,112 can1-100::GFP::CDC11::::TRP1 | This study                         |
| KSC2164         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801 BIR1::::His3 | This study                         |
| KSC2166         | MATa ura3-2 leu2-3,112 ura3-52 his3-2000 lys2-2:His3 lys2-1::::GFP::CDC11::::KAN | This study                         |
| KSC2167         | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801 BIR1::::His3 | This study                         |
|                | GFP::CDC11::::TRP1::::TRP1::::BIR1::::EU2                                  | This study                         |
|                | ndc80-1                                                                  | Wigge et al., 1998                 |
|                | MATa leu2-3,112 ura3 ade2-1 his3-11,15 trp1-1 can1-100 ncdc80-1::::GFP::CDC11::::TRP1::::BIR1::::EU2 | Deshaies and Kirschner, 1995      |
|                | RJD667                                                                   | Biggin et al., 1999                |
|                | leu2 trp1::3 ura3 pep4::::TRP1::::cyc3-4                                   | Gift from Sue Biggins              |
|                | SJY321                                                                   | Goh and Kilmartin, 1993            |
|                | MATa ura3-1 ade2-1 trp1-1::63 his3-11 leu2-3,112 can1-100, ndc10-1         | Basrai et al., 1996                |
|                | YKH8                                                                    | Basrai et al., 1996                |
|                | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801               | Kitagawa et al., 1999              |
|                | YKH10                                                                   | Kitagawa et al., 1999              |
|                | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801               | Sikorski and Hieter, 1989          |
|                | YKK57                                                                   | Connelly and Hieter, 1996          |
|                | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801               | Connelly and Hieter, 1996          |
|                | YKK66                                                                   | Connelly and Hieter, 1996          |
|                | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801               | Connelly and Hieter, 1996          |
|                | YPH1015                                                                  | Connelly and Hieter, 1996          |
|                | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801               | Connelly and Hieter, 1996          |
|                | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801               | Connelly and Hieter, 1996          |
|                | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801               | Connelly and Hieter, 1996          |
|                | MATa ura3-52 lys2-801 ade2-101 trp1-1::63 his3-2000 leu2-801               | Connelly and Hieter, 1996          |
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 μl of TNT mix were used in each 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 factor (American Peptide, Inc.) in medium containing 2% raffinose/galactose. Cells were centrifuged and washed into fresh medium containing a 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; 10× 106 cells were resuspended in 100 μl PBS and supplemented with 4.0 × 105 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 presents 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, sil15, and ipi1 conditional alleles. Fig. S5 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.

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