The integrity of mitosis is dependent upon strict regulation of microtubule stability and dynamics. Although much information has been accumulated on regulators of the microtubule cytoskeleton, our knowledge of the specific pathways involved is still limited. Here we designed genetic screens to identify regulators of microtubule stability that are dispensable in the wild type yet become essential under microtubule-disrupting conditions. We found that the transcriptional cofactor Swi6p and activator Swi4p, as well as the G2/M-specific cyclin Clb2p, are required in a microtubule-destabilizing environment. Swi6p and Swi4p can combine as a transcriptional complex, called the SBF complex (SBF for Swi4/6 cell cycle box [SCB]-binding factor) that is functionally homologous to the metazoan DP1/2-E2F complex and that controls the G1/S transition through the genes it regulates. We show that Swi6p’s contribution to microtubule stability can be either dependent or independent of the SBF complex. The SBF-dependent pathway requires downregulation of SBF complex levels and may thereby reroute the transcriptional program in favor of greater microtubule stability. This pathway can be triggered by overexpression of Fcp1p, a phosphatase in the general transcription machinery, or by expression of an allele of SWI6 that is associated with reduced transcription from SBF-controlled promoters. The SBF-independent pathway is activated by a constitutively nuclear allele of Swi6p. Our results introduce novel roles in microtubule stability for genes whose participation in the process may be masked under normal conditions yet nonetheless acquire a dominant role when microtubule stability is compromised.

The fidelity of chromosome segregation in mitosis is essential for the accurate propagation of genetic information to daughter cells, and it is dependent upon timely, coordinated changes in the microtubule (MT) cytoskeleton. In Saccharomyces cerevisiae, chromosome segregation is achieved in four microtubule-dependent steps. The first step is spindle assembly, which involves the migration of duplicated spindle pole bodies (SPBs) to form a bipolar spindle. This process requires the plus-end-directed activity of the kinesin-like motors of the BimC family, Cin8p and Kip1p, that cross-link and slide antiparallel microtubules of the spindle midzone at the G2/M transition (27, 47). The second step is orientation of the mitotic spindle at the site of cytokinesis, which is mediated by cytoplasmic MTs and requires their transient, dynamic interactions with the cell cortex through dynein-dependent (51) and dynein-independent (50, 4) pathways. The third step is chromosome movement along kinetochore MTs through their depolymerization in anaphase A (46), and the fourth step is complete chromosome separation in the process of anaphase B spindle elongation. This last process is powered by Cin8p and Kip1p that cross-link and push apart polar MTs, by polymerization of the same MTs in the midzone driven by a poleward flux of tubulin subunits, and by a pulling force generated by dynein on cytoplasmic MTs (49).

These changes in the MT architecture are dependent on tight regulation on different levels. Most directly, motor proteins and MT-associated proteins (MAPs) influence MT polymerization, stability, and dynamics (31, 18, 7, 29, 11), thereby affecting processes the specific MT state facilitates. For example, the temperature-sensitive double motor cin8-3 kip1Δ mutant is sensitive to various MT-destabilizing drugs. At elevated temperatures, spindle assembly at G2/M is compromised (20, 42, 49). Introduction of the tub2-402 allele, which hyperstabilizes MTs (28), suppressed the sensitivities both to elevated temperatures and to MT-destabilizing drugs that are associated with this double mutant (39), suggesting destabilized MTs in this background.

Another level of regulation is by upstream cyclin/Cdk complexes whose periodic expression drives specific cell cycle events. Late in G1 phase, the Cin3p-Cdc28p protein kinase complex activates two transcription complexes, the MBF complex (MBF for MluI cell cycle box [MCB]-binding factor) and the SBF complex (SBF for Swi4/6 cell cycle box [SCB]-binding factor), and these in turn promote the transcription of a number of genes important for budding and DNA synthesis (10, 26). At G2/M, the Clb2p-Cdc28p complex represses the activity of SBF, returning the expression of SBF-regulated genes to low levels (1). MT regulation by cyclin/cdk complexes may manifest indirectly: Clb2p-Cdc28p, for example, contributes to the stability or localization of motors posttranslationally (8, 12). It is therefore not surprising that certain Cib/Cdk mutants share the same phenotype exhibited by cin8-3 kip1Δ double mutants grown at the elevated temperature, being unable to assemble a bipolar spindle due to a failure to segregate duplicated SPBs (20, 42, 49). These reports reinforce the ties that exist between upstream and downstream MT regulators.
In wild-type cells under normal conditions, phenotypes are often not manifested because other proteins or pathways act redundantly. A general example is Kss1, a gene whose deletion in wild-type cells has no phenotypic consequence on the mating pathway and therefore was originally thought not to be involved in the process. It was later shown that it can fill in for another protein, Fus3p, which is functionally redundant with Kss1p, when Fus3p is not present (48). Similarly, in this study we aimed to identify proteins that under normal conditions have no apparent role in MT stability, but when the stability of MTs is compromised, their involvement becomes essential.

We previously conducted a genetic screen to identify proteins that when overexpressed can correct the temperature sensitivity and the MT destabilization phenotype associated with cin8-3 kip1Δ cells (54). Here we describe pathways by which Fcp1p, one of the overexpression suppressors, increases sensitivity and the MT destabilization phenotype associated with cells (54). Here we describe pathways by which Fcp1p, one of the overexpression suppressors, increases sensitivity and the MT destabilization phenotype associated with temperature sensitivity and the MT destabilization phenotype associated with temperature sensitivity. Our aim was to identify proteins that underlie the identification of proteins that underlie the identification of the genomic sequences it contains. DNA from transformant colonies was subjected to Southern blot analysis in order to exclude multiple Tn7 insertions.

**Materials and Methods**

Strains and media. *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. All strains are either S288C derivatives or were backcrossed at least seven times to an S288C background. Strain DEY2169 was constructed by crossing strain MAY2169 with strain YNN282 (Yeast Genetic Stock Center, University of California, Berkeley), followed by tetrad dissection and analysis as previously described (32). Medium preparation and yeast genetic and transformation techniques were essentially as previously described (32). Sensitivity to thiabendazole (TBZ) was tested on yeast extract-peptone-dextrose (YPD) agar medium to which the desired amount of TBZ (MP Biomedicals Inc.) was added from 10 mg/ml stock in dimethylformamide (DMF). Serial dilution experiments were repeated three times.

**Screen for multicopy suppressors.** Details of the screen for multicopy suppressors were previously described (54). In brief, after transformation of *cin8-3 dyn1Δ* cells with the YeEp24-based yeast genomic library, colonies that grew at both the permissive temperature (26°C) and the nonpermissive temperature (35°C) upon replica plating were picked. Plasmids were extracted from these colonies, the genomic insert containing the suppressor was sequenced, and the gene responsible for the suppression was isolated. One of these suppressors, the *FCP1* gene, was subcloned into the XbaI and SmaI sites of the 2 μm plasmid YEplac112 and was used to transform *cin8-3 kip1Δ* cells.

**Secondary screen with a Tn7 insertional library, *cin8-3 kip1Δ* double mutants carrying the YeEp112-FCP1 plasmid were transformed with a NotI-digested Tn7-derived insertional library (41). Gene disruptions were generated by homologous recombination as the truncated genomic fragments replaced their native chromosomal locus. In our *cin8-3 kip1Δ* background, such transformations generated triple mutants. Cells containing the transposon were grown at 26°C, replica plated, and incubated at the nonpermissive temperature, 35°C. Since all cells originally grew at the nonpermissive temperature (due to *FCP1* overexpression), colonies that ameliorated this effect subsequent to insertional mutagenesis and grew at 26°C but not at 35°C were selected for further analysis.

**Identification and confirmation of transformants.** To identify genes that ameliorate the effect of *FCP1* overexpression, we isolated genomic DNA from colonies that lost the ability to grow at the nonpermissive temperature, as described previously (32). Thermal asymmetric interlaced (TAIL) PCR (24) was used to amplify flanking sequences adjacent to the insertions. Degenerate PCR primers were as described previously (24), and specific primary and nested primers were designed on the basis of the inserted Tn7 sequences. Sequence data from both ends of the insert were compared against the yeast genome database to determine the identification of the genomic sequences it contains. DNA from transformant colonies was subjected to Southern blot analysis in order to exclude multiple Tn7 insertions.

**Construction of triple mutants.** Deletion strains from the *Saccharomyces* Deletion Project were propagated on YPD plates containing 200 μg/ml Geneticin

| Strain or plasmid | Genotype | Source or reference |
|-------------------|----------|---------------------|
| Strains | | |
| MAYS89 | MATA his3Δ200 leu2-3,112 ura3-52 ade2-101 | M. A. Hoyt |
| MAY2169 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 | M. A. Hoyt |
| YNN282 | MATA his3Δ200 ura3-52 ade2-101 his2-01/01 trp1Δ | YGSC |
| DEY2169 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ | This study |
| DEY3001 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ swi6::KanMX4 | This study |
| DEY3002 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ swi6::KanMX4 | This study |
| DEY3003 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ mbp1::KanMX4 | This study |
| DEY3004 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ chl1::KanMX4 | This study |
| DEY3005 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ chl2::KanMX4 | This study |
| DEY3006 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ chl3::KanMX4 | This study |
| DEY3007 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cbl1::KanMX4 | This study |
| DEY3008 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cbl2::KanMX4 | This study |
| DEY3009 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cbl3::KanMX4 | This study |
| DEY3010 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cbl4::KanMX4 | This study |
| DEY3011 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cbl5::KanMX4 | This study |
| DEY3012 | MATA cin8-3 kip1::HIS3 his3Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cbl6::KanMX4 | This study |
| DEY3020 | MATA his3Δ200 ura3-52 ade2-101 trp1Δ swi6::KanMX4 | This study |
| DEY3030 | MATA his3Δ200 ura3-52 ade2-101 swi4::KanMX4 | This study |
| DEY3001 | MATA his3Δ200 ura3-52 ade2-101 cbl2::KanMX4 | This study |

- Yeast Genetic Stock Center, University of California, Berkeley, CA.

- New England Biolabs

- ATCC

- M. A. Hoyt

- This study

- L. Breeden

- L. Breeden

- M. A. Hoyt

| Plasmids | Genotype | Source or reference |
|----------|----------|---------------------|
| YEplac112 | 2μm TRP1 | New England Biolabs |
| pRS315 | LEU2 CEN | ATCC |
| pMA1208 | CIN8 LEU2 CEN | M. A. Hoyt |
| YEplac112-FCP1 | FCPI 2μm TRP1 | This study |
| BD1265 | SWI6 LEU2 CEN | L. Breeden |
| BD1436 | swi6-S160D LEU2 CEN | L. Breeden |
| BD1651 | swi6-S160A LEU2 CEN | L. Breeden |

- Yeast Genetic Stock Center, University of California, Berkeley, CA.
(Gibco, Invitrogen). DNA was isolated from these strains as described above, and the genes containing the KanMX4 cassettes were PCR amplified using the primers shown in Table 2. PCR products containing the deletion cassettes were verified by gel electrophoresis and transformed into haploid cin8-3 kip1Δ double mutants carrying the 2μm plasmid YEp1c12-FCP1. DNA was subsequently purified from Geneticin-resistant triple mutants, and a confirmation primer (a few hundred base pairs upstream or downstream of the deletion) together with one of the original primers (Table 2) was used to confirm the correct integration.

**Immunoblots.** Ten-milliliter log-phase cultures were centrifuged, and pellets were resuspended in 1 ml ice-cold Tris-EDTA (TE) buffer. Total protein lysate was prepared by vortexing cells with glass beads in 50 μl yeast extraction buffer (0.6% SDS and 10 mM Tris [pH 7.4] with the addition of 1 μg/ml leupeptin [Sigma], 2 μg/ml aprotinin [Sigma], 1 μg/ml pepstatin [Sigma], and 1 mM phenylmethylsulfonyl fluoride [PMSF] [Sigma]). Sample buffer (50 μl) (125 mM Tris-HCl, 20% glycerol, 4.1% SDS, 5% β-mercaptoethanol) was then added, the solution was spun, and the supernatant was boiled for 5 min. A 5:1 sample was mixed with an equal volume of Laemmli Sample Buffer (Bio-Rad) and loaded on 4 to 12% gradient NuPAGE Novex Bis-Tris minigel (Invitrogen), according to the manufacturer’s instructions and detected using Fujifilm (GE Healthcare) were both used at a 1:5,000 dilution. Rabbit anti-Swi6 antibody (a gift from L. Breeden) and peroxidase-linked goat anti-rabbit secondary antibodies (GE Healthcare) were both used at a 1:5,000 dilution. Rabbit anti-Swi4 antibody (a gift from B. Andrews) and peroxidase-linked goat anti-rabbit secondary antibodies (GE Healthcare) were both used at a 1:5,000 dilution. To visualize the results, blots were developed using Amersham’s ECL detection reagents (GE Healthcare) in accordance with the manufacturer’s instructions and detected using Fujifilm Imagel LAS-4000. Fluorescence quantification and analysis were done using Fujifilm Multi Gauge software. Western blots were repeated three times.

**Statistical analysis.** Data were expressed as means ± standard error of the means (SEM). Data were analyzed using analysis of variance coupled with Holm-Sidak test for multiple pairwise comparison. Data analysis was performed using SigmaStat (Chicago, IL). P values of <0.05 were considered statistically significant.

## Results

**FCP1** is a strong suppressor of the temperature and TBZ sensitivities associated with cin8-3 kip1Δ cells. FCP1 on an overexpression vector was identified in our original screen (54) as a strong suppressor of the temperature and thiabendazole (TBZ) sensitivities of cin8-3 kip1Δ mutants. It restored growth at the restrictive temperature (35°C) and conferred resistance to the MT-destabilizing drug TBZ to levels exceeding that of the isogenic wild-type strain (Fig. 1A and B). cin8-3 kip1Δ cells are also sensitive to the MT-destabilizing drug benomyl and its derivative methyl-benzimidazole-2-yl carbendazim (MBC) (39) (data not shown). FCP1 overexpression rendered both double mutant and wild-type cells more resistant to these latter drugs (not shown). TBZ, however, was the preferred drug for this study, as it showed pronounced differences in the growth profiles for various mutations in Swi6p, a protein that is the primary focus of this work.

Fcp1p (transcription factor IIF [TFIIF]-stimulated C-terminal domain [CTD] phosphatase) is a phosphatase in the general transcription machinery. It specifically dephosphorylates Ser2 of the tandem heptapeptide repeat Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 in the C-terminal domain of the largest subunit (Rbp1p) of RNA polymerase II (RNAPII) (36). This conserved heptapeptide repeats 26 times in yeast and up to 52 times in metazoan CTDs (13). Upon transcriptional termination, Fcp1p-mediated CTD dephosphorylation is required to recycle the polymerase at the end of each round of transcript-
tion, an action that primes it for the next transcription cycle (16).

In order to identify domains, and therefore a specific function of Fcp1p that is necessary for the suppression of the cin8-3 kip1Δ phenotype, we assayed the effects of overexpression of various fcp1 mutant genes on our strain. We separately generated amino acid substitutions of the first and second conserved aspartatic acids of the catalytic amino acid substitutions of the first and second conserved aspartatic acids of the \( \text{fcp1} \) C-terminal region of the SSU72 domain (40).

The results of the mutational analysis of Fcp1p led us to conclude that the effect of FCP1 overexpression on MT stability may be indirect, as it requires the normal function of Fcp1p in the context of general transcription. We therefore hypothesized that FCP1 overexpression, through aberrant transcription, triggers a putative pathway that results in greater MT stability. To test this possibility, we designed a secondary screen with a Tn7 insertional library (see Materials and Methods) to search for genes that are needed for the effect of FCP1 overexpression in the cin8-3 kip1Δ background to manifest. Transposon-mediated truncation of SWI6 in the cin8-3 kip1Δ [FCP1-2µ] background restored the sensitivity of the double mutant to the elevated temperature and exacerbated its sensitivity to the microtubule-destabilizing drug TBZ (data not shown). We confirmed these results with a targeted deletion of SWI6 to verify that it is this specific deletion and not other unforeseen elements of the screen that is responsible for ameliorating the effect of FCP1 overexpression on the temperature and TBZ sensitivity of cin8-3 kip1Δ cells (Fig. 2A and B).

Deletion of SWI6 and SWI4 ameliorates the effect of FCP1 overexpression on MT stability in the cin8-3 kip1Δ background. The results of the mutational analysis of Fcp1p led us to conclude that the effect of FCP1 overexpression on MT stability may be indirect, as it requires the normal function of Fcp1p in the context of general transcription. We therefore hypothesized that FCP1 overexpression, through aberrant transcription, triggers a putative pathway that results in greater MT stability. To test this possibility, we designed a secondary screen with a Tn7 insertional library (see Materials and Methods) to search for genes that are needed for the effect of FCP1 overexpression in the cin8-3 kip1Δ background to manifest. Transposon-mediated truncation of SWI6 in the cin8-3 kip1Δ [FCP1-2µ] background restored the sensitivity of the double mutant to the elevated temperature and exacerbated its sensitivity to the microtubule-destabilizing drug TBZ (data not shown). We confirmed these results with a targeted deletion of SWI6 to verify that it is this specific deletion and not other unforeseen elements of the screen that is responsible for ameliorating the effect of FCP1 overexpression on the temperature and TBZ sensitivity of cin8-3 kip1Δ cells (Fig. 2A and B).

Furthermore, to exclude the possibility that a SWI6 deletion independently introduces added temperature sensitivity to the already compromised strain, we introduced to the cin8-3 kip1Δ swi6Δ [swi6Δ] triple mutant cells wild-type \( CIN8 \) on a plasmid. A rescue of the mutant phenotype (Fig. 2A) suggested that the effect of Swi6p on MT stability is pertinent specifically to the cin8-3 kip1Δ genetic background.

Figure 2B also demonstrates that the single swi6 deletion increases the sensitivity of wild-type cells to TBZ to approximately the same degree as that of the cin8-3 kip1Δ double
mutant and that FCP1 overexpression does not increase the resistance to TBZ of the swi6 single mutant. Thus, FCP1 overexpression may act through Swi6p to stabilize microtubules, as it does not bypass the need for Swi6p under microtubule-destabilizing conditions.

To investigate the role of Swi6p in our system, we assayed genes whose products physically interact with Swi6p for their ability to phenocopy the effect of a SWI6 deletion. Swi6p is a transcription cofactor that forms heteromeric complexes with the DNA-binding proteins Swi4p and Mbp1p to mediate transcription at the G1/S transition (26). It is the regulatory or trans-activator subunit of these complexes. SBF (SCB-binding factor) is the complex containing Swi4p and Swi6p that enhances transcription of G1 cyclin genes via SCB elements (Swi4/6 cell cycle box; CACGAAA). MBF (MCB-binding factor) consists of Mbp1p and Swi6p. This complex enhances the transcription of S-phase cyclin genes as well as genes involved in DNA synthesis and repair. It acts via MCB elements (MluI cell cycle box; ACGCGTAA) and leads to the initiation of a complex transcriptional cascade required for coordinated cell cycle progression (10).

Because of the involvement of Swi6p in both these complexes, we specifically looked into the potential implication of these complexes in the suppression. To do this, we first generated cin8-3 kip1Δ swi4Δ and cin8-3 kip1Δ mbp1Δ triple mutants to see whether these deletions similarly block the effect of FCP1 overexpression on the temperature sensitivity of our strains.

As shown in Fig. 2A, cin8-3 kip1Δ swi4Δ cells, but not cin8-3 kip1Δ mbp1Δ cells, lost the ability to grow at the nonpermissive temperature even in the presence of a multicopy plasmid carrying FCP1. Growth of cin8-3 kip1Δ swi4Δ cells at the elevated temperature was restored only upon introduction of a plasmidborne wild-type copy of CIN8, suggesting that a swi4 deletion does not independently introduce sensitivity to the strain. These results suggest that the SBF complex is a likely candidate through which MT stability is conferred. Cells in which swi4 alone was deleted or cells in which swi4 was deleted in combination with cin8-3 kip1Δ are not sensitive to TBZ; however, the deletion does exacerbate the sensitivity of the double mutant to other microtubule-destabilizing drugs, such as benomyl and its derivative MBC (not shown). The mechanisms of action of the three drugs employed in our screens are similar—they all work by sequestering tubulin subunits, preventing polymerization (14, 55). Variation in the sensitivities of different strains to different drugs was previously attributed to ATP-binding cassette (ABC) transporters that are known to be responsible for drug resistance in fungi (6, 43, 44).

FCP1 overexpression downregulates Swi6p and Swi4p to similar extents in cin8-3 kip1Δ cells. We next performed Western blot analysis to further investigate whether the SBF complex has a role in the suppression. We found that in wild-type cells, FCP1 overexpression does not significantly alter the levels of Swi4p and Swi6p (Fig. 3). cin8-3 kip1Δ cells, however, have elevated levels of these proteins compared to the wild type. When FCP1 is overexpressed in the double mutant, the levels of the two proteins return to roughly wild-type levels. Furthermore, though Swi4p and Swi6p are present in different amounts in cells, overexpression of FCP1 in the cin8-3 kip1Δ background has the outcome of downregulating the proteins similarly (1.61- and 1.55-fold, respectively) relative to double mutants that do not overexpress FCP1.

These results demonstrate that the levels of Swi6p and Swi4p in the cell are affected when MT stability is compromised, as in the cin8-3 kip1Δ background. This compromised condition may be sensed, resulting in a change in transcription patterns. More importantly, and in line with Fcp1p’s role in transcription, the results suggest that in cin8-3 kip1Δ cells, FCP1 overexpression leads to a reduction in the levels of the SBF complex specifically, as its components Swi4p and Swi6p are downregulated comparably. Taken together with the results shown in Fig. 2, the data reveal a correlation between SBF complex levels and MT stability and present the possibility that curtailing transcription from SBF promoters supports MT stability.

Expression of the swi6-S160D allele, which results in reduced transcription from SBF-regulated promoters, rescues the TBZ sensitivity of cin8-3 kip1Δ swi6Δ cells. The results in Fig. 2 and 3 that point to the SBF complex as an intermediary through which MT stability is reconciled led us to ask whether alteration of transcription from SBF promoters is needed for the suppression of the TBZ sensitivity of our mutants. To examine this, we obtained two alleles of SWI6, a constitutively nuclear (swi6-S160A) allele, whose expression does not reveal major changes in SBF-driven transcription, and a predominantly cytoplasmic (swi6-S160D) allele, which was shown to result in a marked reduction of transcription from SBF-regulated promoters (52). Protein expression from plasmids carrying these alleles was previously shown to be comparable to protein expression from a plasmid carrying the wild-type SWI6 gene (52).

We expressed these alleles in the compromised cin8-3 kip1Δ swi6Δ strain. As shown in Fig. 4A, introduction of a wild-type copy of SWI6 on a centromeric plasmid resulted in the predicted complementation of the temperature-sensitive
phenotype. Introduction of other alleles of \textit{SWI6} on a plasmid also resulted in complementation. When assayed for growth on a medium containing TBZ, \textit{cin8-3 \textit{kip}1\textDelta swi6\Delta [\textit{FCP1-2\mu}]\textit{ cells carrying wild-type \textit{SWI6} or swi6-S160A on a plasmid resulted in resistance to the microtubule-destabilizing drug at a level roughly equivalent to that of \textit{cin8-3 \textit{kip}1\Delta} double mutants overexpressing \textit{FCP1} (compare Fig. 4A with Fig. 1B). Interestingly, cells carrying the \textit{swi6-S160D} allele demonstrated even higher resistance to TBZ. This led us to suggest that an additive effect may be in place when both \textit{FCP1} overexpression and \textit{Swi6-S160D} are present in the \textit{cin8-3 \textit{kip}1\Delta swi6\Delta} background, resulting in such high levels of resistance. If this is the case, then the \textit{swi6-S160D} allele may be able to mediate sufficient suppression in the absence of \textit{FCP1} overexpression.

To test this hypothesis, triple mutants were cured of the multicopy plasmid carrying \textit{FCP1}. Figure 4B reveals that while plasmids carrying the wild-type \textit{SWI6} or its \textit{S160A} version no longer supported growth at TBZ concentrations that compromised the double mutant, \textit{swi6-S160D}, although less resilient at extremely high TBZ concentrations in the absence of \textit{FCP1} overexpression, is nonetheless able to restore growth in the cells to a level similar to that of the double mutant overexpressing \textit{FCP1} (compare Fig. 4B with Fig. 1B).

These results suggest that the effects of these \textit{swi6} alleles on MT stability is independent of \textit{FCP1} overexpression and may substitute for it to achieve the same suppression. Furthermore, because of its reported reduced transcription from SBF promoters (52), the suppression caused by the \textit{S160D} allele of \textit{SWI6} proves that not only is modulation of the levels of the proteins that make up the SBF complex needed (as suggested in Fig. 3) but that a functional SBF complex must be in place to exert its effect on transcription of the \textit{G1/S} genes it is known to regulate.

Expression of the constitutively nuclear \textit{swi6-S160A} allele causes resistance to elevated temperatures for \textit{cin8-3 \textit{kip}1\Delta swi6\Delta} cells. In the presence of \textit{FCP1} overexpression, all alleles of \textit{SWI6} tested have the capacity to complement the temperature-sensitive phenotype (Fig. 4A). However, in the absence of \textit{FCP1} overexpression, only the \textit{swi6-S160A} allele is able to suppress the temperature sensitivity of our mutants (Fig. 4B).

The fact that rescue of the temperature sensitivity in the absence of \textit{FCP1} overexpression requires the \textit{S160A} allele while resistance to TBZ requires the \textit{S160D} allele of \textit{SWI6} is explained as follows: when damage to microtubules is global (i.e., when both nuclear and cytoplasmic MTs are affected, as is the case when cells are grown in the presence of TBZ), downregulating \textit{G1/S} transcription from SBF-controlled promoters through expression of the \textit{swi6-S160D} allele can suppress the MT defect. On the other hand, when the damage is limited to spindle MTs, as is the case for \textit{cin8-3 \textit{kip}1\Delta} cells grown at the elevated temperatures (20, 42, 49), the constitutive presence of Swi6p in the nucleus can cause some suppression. There may be a dose effect in that Swi6p-\textit{S160D}, which is mostly cytoplasmic, is not able to achieve the same effect at elevated temperatures when the damage is solely to spindle MTs. Since expression of the \textit{swi6-S160A} allele does not alter transcription from SBF-regulated promoters (52), this suggests that there may be another role for Swi6p in MT stability that is distinct from its role in the SBF complex.

\textbf{Deletion of \textit{CLB2} abrogates the \textit{Fcp1p-mediated suppression of the temperature and TBZ sensitivities of \textit{cin8-3 \textit{kip}1\Delta} cells.} To explore the possibility that regulators of the SBF complex are also involved in \textit{FCP1's} suppression and therefore relevant to our background, we screened different cyclins for their ability to mitigate the effect of \textit{FCP1} overexpression on MT stability in the \textit{cin8-3 \textit{kip}1\Delta} background. The rationale for looking into cyclins is that Cin8p, Kip1p, Fcp1p, Swi6p, and Swi4p were all previously shown to physically interact with Cdc28p, the catalytic subunit of the main cyclin-dependent kinase (1, 8, 22, 56), which pairs up with different cyclins to regulate cell cycle progression in yeast. Furthermore, strains containing certain cyclin or \textit{CDC28} deletions are incapable of assembling a bipolar spindle, a phenotype shared with our double mutant (20).

As the periodic expression of cyclins serves to limit the window of action of Cdc28p to the proper time in the cell cycle.
mechanisms by which upstream cell cycle regulators affect the stability of the microtubule cytoskeleton.

**FCP1 overexpression and MT stability.** FCP1 is an essential gene in both budding and fission yeast and is conserved among eukaryae (2). Domain analysis of Fcp1p revealed that both the phosphatase activity and functional targeting to the transcription machinery are required for the suppression of the temperature sensitivity of *cin8-3 kip1Δ* cells (Fig. 1C). These data, together with the established role of Fcp1p in global gene transcription, suggest that the effect of *FCP1* overexpression on MT stability may not be direct (i.e., mediated through physical interaction with components of the cytoskeleton) but instead may be indirect, possibly involving different pathways that act in conjunction or in parallel. Beyond the role of Fcp1p in transcription, it may be a key player in shaping specific phosphorylation patterns in its substrate, the C-terminal domain of the large subunit of RNAII, through dephosphorylation of Ser-2 in the heptad repeats of the CTD. The significance of the phosphorylation state of the CTD of RNAII is iterated in the proposed “CTD code” (5), which was suggested to convey information to CTD-binding proteins, some of which recognize particular phosphorylation patterns and trigger specific cellular responses (19).

How can overexpression of *FCP1* suppress defects associated with a compromised microtubule cytoskeleton? Mutations in *FCP1* increase phosphorylation of Ser2 residues of the CTD of RNAII and rapidly shut down mRNA synthesis (30, 37). Conversely, *FCP1* overexpression in *Schizosaccharomyces pombe* results in increased levels of the hypophosphorylated (IIa) form of the CTD relative to control strains (35). By overexpressing *FCP1* in our strains, we may be mimicking massive dephosphorylation of the CTD. It was demonstrated in *Xenopus* extracts that upon fertilization, the CTD undergoes massive dephosphorylation of the CTD. The transition results in the activation of intracellular signals, some of which regulate changes in the microtubule cytoskeleton, including remodeling of the MT architecture that is required for expulsion of half the chromosomes into the polar body and for the establishment of a cytoplasmic microtubule network whose responsibility is to promote migration of the male and female pronuclei (21). Likewise, *FCP1* overexpression in our genetic background may specify a transcriptional program that is accompanied by changes in MT stability.

**SBF-dependent route to MT stability.** The transcriptional program specified by *FCP1* overexpression that is relevant for MT stability converges with Swi6p, Swi4p, and Cib2p, because deletion of these genes, separately, in the *cin8-3 kip1Δ* background abrogates the *FCP1*-mediated suppression of the temperature sensitivity and the sensitivity to MT-stabilizing drugs that are associated with these cells.

The relevance of Cib2p for MT-dependent processes was previously characterized. Cells in which *CLB2* has been deleted have a diminished capacity to separate the SPBs (20), a phenotype in common with *cin8-3 kip1Δ* double mutants grown at the nonpermissive temperature after release from α-factor-induced *G1* arrest (27, 47, 49). Additionally, the cyclin/Cdk

**DISCUSSION**

This study suggests novel roles in MT stability for proteins that were not previously associated with this function. It opens new avenues of research into the exploration of the specific
complex Clb2/Cdc28 was shown to promote SPB separation and spindle assembly via Cin8p and Kip1p. The motors are phosphorylated directly by the Clb2/Cdc28 complex in vitro and in vivo, and this phosphorylation plays a role in promoting SPB separation and spindle assembly (8). In another study, it was proposed that Clb2/Cdc28 kinase activity regulates SPB separation indirectly by preventing the degradation of the motors (12).

Still, there may be paths to MT stability mediated through Clb2p that were not previously considered, paths that become dominant when the MT cytoskeleton is compromised. For example, the Clb2/Cdc28 complex, by virtue of its interaction with and phosphorylation of Swi4p, was shown to switch off SBF-dependent transcription at the G2/M phase (1, 38), a time when cin8-3 kip1Δ cells become arrested at the restrictive temperature (27, 47). In our study, the presence of FCP1 on a multicopy plasmid correlated with a proportional reduction in the levels of Swi6p and Swi4p relative to cells not overexpressing FCP1 (Fig. 3), suggesting that the SBF complex is being downregulated, and expression of the swi6-S160D gene, which leads to reduced activity from SBF promoters (52), was able to substitute for FCP1 overexpression in suppressing the TBZ sensitivity. Because of this similar effect of Clb2p at G2/M, of FCP1 overexpression, and of constitutive expression of swi6-S160D on the SBF complex and on MT-dependent processes, it is not unreasonable to propose that when MT stability is compromised, downregulation of the SBF complex alleviates the impairment. This downregulation, in turn, may lead to rerouting of the transcriptional program in favor of greater MT stability.

SBF-independent route to MT stability. Several lines of evidence led us to propose the notion of an SBF-independent route to MT stability. The first is the suppression of the temperature sensitivity of cin8-3 kip1Δ swi6Δ cells by constitutive expression of the S160A allele of SWI6 (Fig. 4B), an allele which does not lead to major changes in cell cycle-specific activation or repression of transcription of SBF-regulated promoters (52). The second is the fact that deletion of regulators of the SBF complex did not achieve the same abrogation of FCP1 suppression, as did a deletion of SWI4 or SWI6 (Fig. 5A).

Swi6p shuttles in and out of the nucleus in a cell cycle-dependent manner, with nuclear import occurring concomitantly with dephosphorylation of Ser-160 in late mitosis and with nuclear export peaking from late G1 to M phase and requiring phosphorylation of Ser-160 (22). Activation or repression of Swi6p-regulated genes is independent of Swi6p's phosphorylation and hence, its localization (52). Aspartate substitution of serine 160 was shown to significantly impair phosphorylation and hence, its localization (52). Aspartate substitution at the same position leads to constitutive nuclear localization, causing Swi6p to remain predominantly cytoplasmic throughout the cell cycle (though Swi6p is not completely excluded from the nucleus), whereas an alanine substitution at the same position leads to constitutive nuclear accumulation of Swi6p (52).

Interestingly, there is another convergence point between Clb2p and Swi6p which is independent of the SBF complex but dependent on the principal mitotic exit regulator Cdc14p. Clb2/Cdc28 was shown to lead to the liberation of Cdc14p from nucleolar sequestration in early anaphase (3). At anaphase onset, when the spindle starts to elongate, the phosphatase activity of Cdc14p was shown to be required for nuclear microtubule stabilization (25) and for the localization of spindle midzone proteins like Cin8p (34). When CLB2 is deleted, cells are defective for early anaphase release of Cdc14p (3), and cdc14-1 clb2::LEU2 double mutants do not start anaphase and arrest with unseparated spindle pole bodies (57), an arrest phenotype shared with cin8-3 kip1Δ cells cultured at the nonpermissive temperature after release from G1 synchronization (20). Recently, it was shown that CDC14 overexpression can bring about SPB separation in cin8-3 kip1Δ cells (9).

We therefore propose that in cin8-3 kip1Δ cells, Swi6p may have the potential to mediate the role of Cdc14p in nuclear MT stabilization through a similar mechanism to that by which Cdc14p leads to an increase in nuclear MT stability in wild-type cells (25). Cdc14p normally recruits Swi6p to the nucleus only in late M phase (22), but constitutive nuclear localization of Swi6p (through expression of the S160A allele) may be able to stabilize spindle MTs in the compromised cin8-3 kip1Δ background. This is supported by our finding that the nuclear version of Swi6p restores growth at the nonpermissive temperature independently of FCP1 overexpression (Fig. 5B).

Our results demonstrating that certain SWI6 alleles play a part in MT stability raise the possibility that under normal conditions, transient expression of different phosphorylated forms and their restriction to specific cell cycle phases serves to limit the functional diversity of Swi6p. Constitutive expression of modified forms of Ser-160 may bring out Swi6p's MT-stabilizing potential.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (grant GM065885) and the Professional Staff Congress of the City University of New York.

We thank P. Lipke, S. Krishnamurthy, A. Kumar, L. Breeden, B. Andrews, and C. Forest for donating strains, plasmids, antibodies, primers, and other materials for this work, P. Lipke and R. Tal for critical comments on the manuscript, and R. Tal and E. Korolyev for technical assistance.

REFERENCES

1. Amon, A., M. Tyers, B. Butler, and K. Nasmyth. 1993. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. Cell 74:993–1007.
2. Archambault, J., et al. 1997. An essential component of a C-terminal domain phosphatase that interacts with transcription factor IIF in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. 94:14300–14305.
3. Azzam, R., et al. 2004. Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. Science 305:516–519.
4. Berlin, V., C. A. Styles, and G. R. Fink. 1990. BYK1, a protein required for microtubule function during mating and mitosis in Saccharomyces cerevisiae, colocalizes with tubulin. J. Cell Biol. 111:2573–2586.
5. Buratowski, S. 2003. The CTD code. Nat. Struct. Biol. 10:679-680.
6. Cabanas, R., G. Castella, M. L. Abarca, M. R. Bragulat, and J. F. Cahanes. 2009. Thiabendazole resistance and mutations in the beta-tubulin gene of Penicillium expansum strains isolated from apples and pears with blue mold decay. FEMS Microbiol. Lett. 297:189–195.
7. Carminati, J. L., and T. Stearns. 1997. Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex. J. Cell Biol. 138:629–641.
8. Chee, M. K., and S. B. Haase. 2010. B-cyclin/CDKs regulate mitotic spindle assembly by phosphorylating kinesin-5 in budding yeast. PLoS Genet. 6:e1000935.
9. Chirolli, E., G. Rancati, I. Catusi, G. Lucchini, and S. Piatto. 2009. Cdc14 inhibition by the spindle assembly checkpoint prevents unscheduled centrosome separation in budding yeast. Mol. Biol. Cell 20:2626–2637.
35. Kaiser, C. M., and S. A. Mitchell.
31. Kamenski, T., S. Heilmeier, A. Meinhart, and P. Cramer.
34. Khmelinskii, A., C. Lawrence, J. Roostalu, and E. Schiebel.
30. Huyett, A., J. Kahana, P. Silver, X. Zeng, and W. S. Saunders.
29. Hoyt, M. A., L. He, K. K. Loo, and W. S. Saunders.
22. Gallicano, G. I.
24. Fitch, I., et al.
23. Fabrega, C., V. Shen, S. Shuman, and C. D. Lima.
27. Hoyt, M. A., L. He, K. K. Loo, and W. S. Saunders.
26. Gonzalez-Ballester, D., A. de Maontaigu, A. Galvan, and E. Fernandez.
20. Rutherford, S. L. 2000. From genotype to phenotype: buffering mechanisms and the storage of genetic information. Bioessays 22:1095–1105.
19. Saunders, W. S., D. Koshland, D. Estel, I. R. Gibbons, and M. A. Hoyt. 1995. Saccharomyces cerevisiae kinases- and dynein-related proteins required for anaphase chromosome segregation. J. Cell Biol. 128:617–624.
18. Shaw, S. L., E. Yeh, P. Maddox, E. D. Salmon, and K. Bloom. 1997. Astral microtubule dynamics in yeast: a microtubule-based searching mechanism for spindle orientation and nuclear migration into the bud. J. Cell Biol. 139:985–994.
17. Sidorova, J. M., G. E. Mikesell, and L. L. Breeden. 1995. Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization. Mol. Biol. Cell 6:1616–1628.
16. Solomon, F., C. L. D. Kirkpatrick, V. Pratiss, and B. Weinstein. 1992. Methods for studying the yeast cytokinesis, p. 197–222. In K. Carraway and C. Carraway (ed.), The cytokinesis. Oxford University Press, Oxford, United Kingdom.
15. Steinberg-Neifach, O., and D. Estel. 2000. Simultaneous expression of both MAT loci in haploid cells suppresses mutations in yeast microtubule motor genes. Mol. Gen. Genet. 264:300–305.
14. Walker, G. M. 1982. Cell cycle specificity of certain antimicrotubular drugs in Schizosaccharomyces pombe. J. Gen. Microbiol. 128:61–71.
13. Wong, J. et al. 2007. A protein interaction map of the mitotic spindle. Mol. Biol. Cell 18:3800–3809.
12. Yuste-Rojas, M., and F. R. Cross. 2000. Mutations in CDC41 result in high sensitivity to cyclin gene dosage in Saccharomyces cerevisiae. Mol. Gen. Genet. 263:60–72.
11. Cottingham, F. R., and M. A. Hoyt. 1997. Mitotic spindle positioning in Saccharomyces cerevisiae II: correlation of artificially augmented microtubule motor proteins. J. Cell Biol. 138:1041–1053.
10. Costanzo, M., O. Schuh, and B. Andrews. 2003. G1 transcription factors are differentially regulated in Saccharomyces cerevisiae by the Swi6-binding protein Stb1. Mol. Cell. Biol. 23:5064–5077.
9. Kumar, A., et al. 2004. Large-scale mutagenesis of the yeast genome using a Tet-derived multipurpose transposon. Genome Res. 14:1975–1986.
8. Lim, H. H., P. V. Goh, and U. Surana. 1996. Spindle pole body separation in Saccharomyces cerevisiae requires dephosphorylation of the tyrosine 19 residue of Cdc28. Mol. Cell. Biol. 16:6385–6397.
7. Nakaune, R., et al. 1998. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus Penicillium digitatum. Appl. Environ. Microbiol. 64:3983–3988.
6. Nakaune, R., H. Hamamoto, J. Imada, K. Akutsu, and T. Hibi. 2002. A novel ABC transporter gene, PMR5, is involved in multidrug resistance in the phytopathogenic fungus Penicillium digitatum. Mol. Genom. Genomes 267:179–185.
5. Palancade, B., M. F. Dubois, M. E. Dahmus, and O. Bensaude. 2001. Transcription-independent RNA polymerase II dephosphorylation by the FCP1 carboxy-terminal domain phosphatase in Xenopus laevis early embryos. Mol. Cell. Biol. 21:6359–6368.
4. Peterson, J. B., and H. Ris. 1976. Electron-microscopic study of the spindle and chromosome movement in the yeast Saccharomyces cerevisiae. J. Cell Sci. 22:219–242.
3. Roof, D. M., P. B. Meluh, and M. D. Rose. 1992. Kinase-related proteins required for assembly of the mitotic spindle. J. Cell Biol. 118:905–108.
2. Rutherford, S. L. 2000. From genotype to phenotype: buffering mechanisms and the storage of genetic information. Bioessays 22:1095–1105.