REGULATION OF MICROTUBULE STABILITY IN *SACCHAROMYCES CEREVISIAE*

By

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Abstract

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The integrity of chromosome segregation during mitosis is essential for the propagation of genetic information to daughter cells during cell division. In yeast, it is achieved in four microtubule-dependent steps: first is spindle assembly, which involves the migration of duplicated microtubule organizing centers to form a bipolar spindle by prophase; second is orientation of the mitotic spindle at the site of cytokinesis; third- chromosome movement along kinetochore microtubules (anaphase A); and fourth, complete chromosome segregation through spindle elongation through interpolar microtubules (anaphase B). Signaling pathways have been implicated in the regulation of microtubule dynamics and stability, which is required for these processes. This work identifies additional protein regulators of microtubule stability using mutants of key mitotic motor proteins, specifically, Cin8p, Kip1p, and Dyn1p. Loss of Cin8p function in the absence of either Kip1p or Dyn1p is lethal. Haploid cells that carry the cin8-3 temperature sensitive allele in a deletion background of either *KIP1* or *DYN*1 cannot grow above 35◦C. Our studies suggest that suppressors of these mutant genotypes act by stabilizing microtubules. We propose that the mechanism of suppression involves enhancing signal-transduction cascades that regulate microtubule stability and dynamics. We found that

FCP1 overexpression supressess the microtubule defect in our background and that this suppression requires the following genes to manifest: *SWI6*, *SWI4*, *CLB2*, *ELM1*, *HSL1* and *MRS6*. These proteins, with a previously uncharacterized role in microtubule stability, may be candidate microtubule-associated proteins (MAPs) or novel regulators of MAPs (direct or indirect). In addition, a putative pathway to MT stability was drawn based on genetic interactions we established, epistatic experiments that were done and physical data we produced, combined with existing knowledge.

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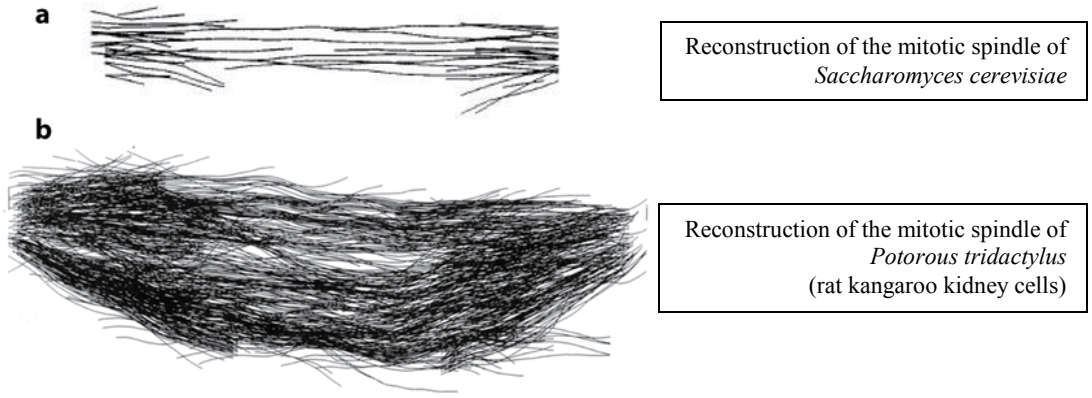
CHAPTER I

INTRODUCTION

1.1 A CASE FOR BUDDING YEAST AS A MODEL TO STUDY MICROTUBULE REGULATION

We focus on budding yeast as the primary organism in which to study processes relevant to mitosis for the simplicity of its design. The entire yeast spindle is comprised of approximately 40 microtubules (32 kinetochore microtubules and 8 interpolar microtubules, which will be described in detail in the following sections), versus up to 100 times that in certain mammalian cells[1] (see Figure 1.1).

Figure 1.1 Comparison of the mitotic spindle in yeast and mammals



Bouck DC, et al. 2008.
Annu. Rev. Genet. 42:335–59

Furthermore, only one microtubule attaches at each kinetochore, alleviating the complication of dealing with microtubule bundles. This extraordinary simplicity of the budding yeast spindle allows the role of microtubules as a mechanical element of the mitotic spindle to be examined with relative ease. From a mechanical perspective, the simplicity provides us with an opportunity to evaluate the relative contributions of individual proteins or events, and connections between structures can be easily measured and assessed. Moreover, the basic principles deduced from the function of this primitive

spindle are highly conserved throughout the phylogeny and are applicable to understanding both the structure and function of mitotic spindles in all eukaryotes.[1]

Additionally, Yeast is exceedingly amenable to genetic manipulations. Site-specific integration of foreign DNA into the genome by transformation to allow for high efficiency gene knockouts is commonly practiced, as well as gene replacements and gene tagging, taking advantage of endogenous homologous recombination mechanisms. In fact, generation of strains containing knockouts or alleles of different genes is also greatly facilitated as yeasts are viable with many markers and quite a few nonessential genes. Structural genes corresponding to virtually any genetic trait can be identified by complementation from plasmid libraries.

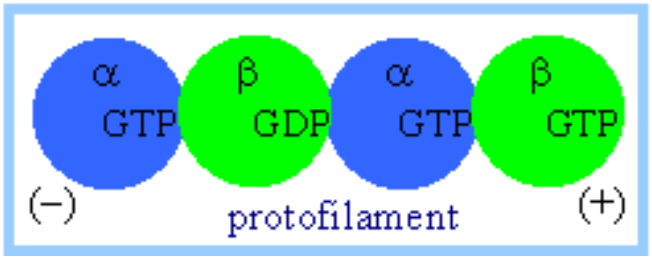
Finally, yeasts can exist stably in either diploid or haploid states and can be easily switched between these two states, which further simplifies genetic analysis of various mutants. All these techniques have been used in this current study and have shed much light on the involvement of distinct genes in the stability of the microtubule cytoskeleton.

1.2 MICROTUBULE STRUCTURE AND POLARITY

Microtubules (MTs) are dynamic polymers that alternate stochastically between phases of slow growth and rapid shrinkage, a property known as dynamic instability[2]. The four known MT-dependent processes in yeast are mitosis, meiosis, and karyogamy (mating), as well as spindle positioning in preparation for the first three. These activities are associated with a dynamic restructuring of the MT cytoskeleton, for example, lengthening and shortening of individual MTs or even complete disassembly and rebuilding of MT arrays[2].

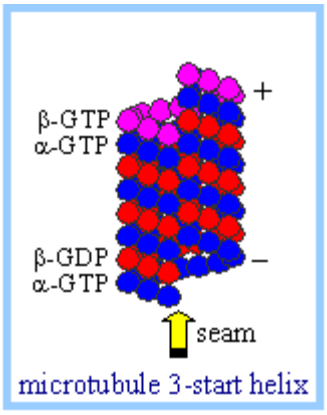
An α,β-tubulin heterodimer is the basic structural unit of microtubules and the heterodimer does not come apart once formed. The α and β tubulins, with a molecular weight of about 55 kDa each, are homologous but not identical. Each has a nucleotide binding site: α-tubulin has a bound molecule of GTP that does not hydrolyze, while β-Tubulin may have bound GTP or GDP. Under certain conditions β-tubulin can hydrolyze its bound GTP to GDP plus P_i, release the P_i and exchange the GDP for GTP[2] (see Figure 1.2).

Figure 1.2 Protofilament structure. Adapterd from Alberts B, Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P.: Molecular Biology of The Cell, 2006



In microtubules, self-assembling αβ- tubulin heterodimers are arranged longitudinally to form protofilaments. 13 protofilaments are laterally attached, forming hollow tubes that are approximately ~24 nm in diameter. The alignment of tubulin dimers within MTs leaves α-tubulins exposed at one end and β-tubulins exposed at the other which gives MTs intrinsic structural and kinetic polarity, as the rate of polymerization is faster at the plus end, where the β-tubulins are exposed, and the slower growing (minus) end where α-tubulins are exposed[3] (see Figure 1.3).

Figure 1.3 Microtubule structural polarity. Adapterd from Alberts B, Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P.: Molecular Biology of The Cell, 2006



Subunit addition brings β-tubulin exposed at the plus end of a microtubule into contact with α-tubulin of an incoming heterodimer. The GTP on α-tubulin does not hydrolyze, yet α-tubulin was suggested to contribute an essential residue to the catalytic site of β-tubulin, possibly serving as a GTPase activating protein (GAP) for β-tubulin of the adjacent dimer in a protofilament. This contact promotes hydrolysis of GTP bound to the now interior β-tubulin. P_i dissociates, but β-tubulin within a microtubule cannot exchange its bound GDP for GTP[3].

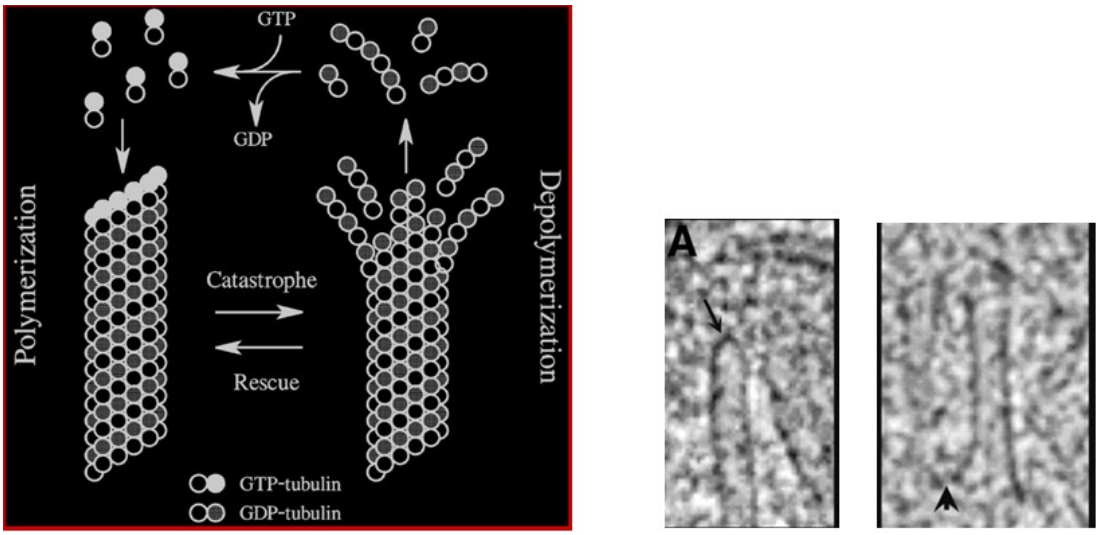
1.3 MICROTUBULES & DYNAMIC INSTABILITY

Microtubule dynamics are described in terms of the velocity of MT growth and shortening and the frequency of switching between these two states (rescue is the transition from shortening to growth; catastrophe is the switching from growth to shortening)[1].

Microtubules may grow steadily and then shrink rapidly by loss of tubulin dimers at the plus end. The transition between growth and shrinkage, which occurs through a continuous exchange of tubulin subunits between monomer pools and plus-ends of MT filaments, is thought to be controlled by the structure of MT ends[4]. When tubulin dimers add to a growing (+) end, β-tubulin bound GTP is hydrolyzed as polymerization brings it into contact with α-tubulin. Hydrolysis, however, takes time, so that a rapidly growing microtubule may accumulate a few layers of tubulin-GTP at the plus end. This creates a 'GTP cap' that closes to form a tube which prevents MTs from depolymerizing. On a biophysical level, this is achieved by preventing the unstable GDP-tubulin within the lattice from adapting its preferred outwardly curved conformation[5].

As microtubules grow, tubulin dimers are depleted. Below a critical concentration of heterodimers, rapid shrinkage at the plus end ensues which has been attributed to loss of this stabilizing GTP cap. Tubulin-GTP start dissociating and expose tubulin-GDP at the plus end, causing that end to become unstable (i.e., splayed, such that protofilaments bend back toward the shaft of MTs[6]). The fraying or curving of protofilaments observed at the ends of rapidly disassembling microtubules is due to a change in conformation when β-subunits at the plus end have bound GDP instead of GTP. The release of free energy that accompanies relaxation of the strained GDP-tubulin is thought to promote rapid shrinkage, or catastrophe[7] (see Figure 1.4).

Figure 1.4 MT end morphology. Left: Dynamic instability. Below: EM micrographs of MT minus ends (left) that are capped and often appear pointed or tapered, and MT plus ends (right) that often have a distinct flaring. From O'Toole *et al.,* 1999.



1.4 MICROTUBULE-ASSOCIATED PROTEINS

The only two levels of MT organization observed *in vitro* are ring-shaped assemblies of tubulin heterodimers when GDP is bound to the β–subunit, or straight protofilaments when both tubulins have bound GTP[8]. In cells, however, the microtubule cytoskeleton undergoes timely, coordinated, and specific changes in organization and function, suggesting that microtubule dynamics *in vivo* are regulated rather than simply being driven by an equilibrium between polymerized and non-polymerized states. This regulation is both spatial and temporal: it is evident at various points in the cell cycle and is characteristic of distinct cellular processes that are MT-dependent. For example, the aster-like array characteristic of cytoplasmic microtubules in the G1/S phase interconverts to a rigid elongating spindle during mitosis that is responsible for segregating the

chromosomes during division. Furthermore, this interconversion is coordinated with the growth of the developing bud.

In meiosis, the morphology assumed by the meiotic spindle allows it to participate in both the reductional and equational meiotic divisions necessary to generate haploid progeny [9]. During mating MTs rearrange to bring nuclei of zygotes together in the karyogamy process[10, 11]. Specifically, before the fusion of cells of opposite mating types, the microtubule cytoskeleton in each of the mating cells reorients so that the cytoplasmic microtubules are projected in the direction of the mating partner. Once the cell walls and plasma membranes have fused, the cytoplasmic microtubules associated with each nucleus function in a coordinated fashion to achieve nuclear fusion of the newly formed zygote. As the diploid cell reinitiates mitotic growth, the organization and function of the microtubules are again reorganized to follow the mitotic program[12]. These observations imply the existence of factors in cells that affect MT stability and dynamics.

In *S. cerevisiae*, there is only a single β-tubulin gene, *TUB2*, and two α-tubulin genes, *TUB1* and *TUB3*; therefore, it is reasonable to assume that the complex and coordinated changes of the microtubule cytoskeleton during mitosis, meiosis, and mating cannot be attributed solely to this limited heterogeneity of the tubulin proteins, but may more likely be due to activities of *trans*-acting factors. Microtubule-associated proteins, or MAPs, were originally identified as proteins that co-pellet with assembled mictorubules in mammalian brain tubulin cycling experiments[13]. MAPs were later revealed to bind polymerized MTs or tubulin dimers, altering the frequency of catastrophe and the rate of polymer rescue[14]. Furthermore, the binding of some MAPs to microtubules was shown to be regulated by phosphorylation, providing another level of

regulation necessary for such dramatic restructuring of the cytoskeleton that occurs throughout the cell cycle.

MAPs may play a structural role and participate directly in an assembly or disassembly process. Alternatively, MAPs may regulate a critical step in the assembly or disassembly process, or in the duration of growth and shortening events. MAPs may stabilize a MT by preventing catastrophic shrinkage or by promoting microtubule growth. Their binding to MTs may stimulate the addition of tubulin dimers at the plus end or inhibit catastrophe. Alternatively, MAPs may promote the dissociation of tubulin dimers, in which case they would be considered catastrophins, or catastrophe-promoting proteins. The mechanism for destabilization may be through the activation of GTP hydrolysis or by inducing a curved protofilament conformation. They may also bind to tubulin heterodimers, decreasing their availability for polymerization. Such depletion of MT building blocks would result in heterodimer dilution to levels below the critical concentration, shifting the equilibrium between the polymerized and depolymerized state in favor of depolymerization. MAPs may also physically sever the lattice, generating new plus ends that lack a stabilizing GTP cap and minus ends that are not stabilized by being capped by γ-ring complexes of the microtubule-organizing center. They may also interfere with capping by associating with uncapped minus ends of microtubules[8].

Other than affecting MT polymerization, MAPs may play a role in mediating interactions between MTs and other cell constituents, guiding them towards specific cellular locations, crosslinking them, or may be involved in some yet unidentified roles[12].

MAPs in S. cerevisiae

The microtubule-associated proteins in yeast are critical for spindle structure and stability. They include +TIPS (plus-end tracking proteins), cortical anchorage components (which mediate interaction between MTs and the cell cortex underlying the plasma membrane), and microtubule-associated motors.

+TIPS decorate microtubule plus-ends and regulate microtubule polymerization dynamics, as well as the duration of growth and shortening events, some of which are critical for the structure and stability of the mitotic spindle as a whole. An example is the 10-subunit Dam1/DASH protein complex which facilitates kinetochore-MT attachment[15]. MAPs at this complex are suggested to promote dissociation of tubulin dimers at the poles of the cell, contributing to treadmilling (flow of microtubule subunits toward the poles) and shortening of kinetochore-linked microtubules during anaphase. Bik1p, another +TIP, contributes to the maintenance of depolymerizing MTs at sites of cortical growth in mating yeast[16]. In both instances, once the plus end of a microtubule makes contact with a chromosome or the cell cortex, it becomes stabilized or destabilized by assembly or rapid disassembly (respectively), events which are catalyzed at the plus end by the particular MAP[1].

Cortical anchorage components, such as Bim1p and Kar9p, make up the cytoplasmic microtubule capture site at the cell cortex[17]. They help with spindle positioning by promoting MT dynamicity during the G_1 phase of the cell cycle (dynamic MTs increase the probability of a MT end encountering a capture site[18]). These proteins also have a role in delaying mitotic exit when the spindle is oriented abnormally[1].

Other MAPs exert their effect on MTs by binding to the MT lattice. Ase1p, Stu1p and Slk19p decorate overlapping antiparallel microtubules of the spindle midzone (which will be discussed in the following section) and have roles in spindle elongation and stabilization[19]. Some of these proteins, termed passengers (i.e., Ndc10p), are proteins that relocate from the centromere to the spindle midzone following anaphase onset. Their deletions or mutations result in phenotypes including spindle stability defects and delayed spindle disassembly[1]. Finally, motor proteins too exercise their power on the mitotic spindle by binding to the MT lattice.

1.5 THE YEAST MICROTUBULE-ORGANIZING CENTER AND THE MITOTIC SPINDLE

The yeast microtubule-organizing center (MTOC) is a dynamic, disk shaped organelle called the spindle pole body or SPB, that consists of three plaque structures serve as the yeast homologues of centrioles: an outer plaque exposed to the cytoplasm, which anchors the first class of MTs- cytoplasmic or astral microtubules, a central plaque that anchors the SPB in the nuclear envelope, and an inner plaque facing the nucleoplasm, that anchors nuclear MTs[20] (see Figure 1.5).

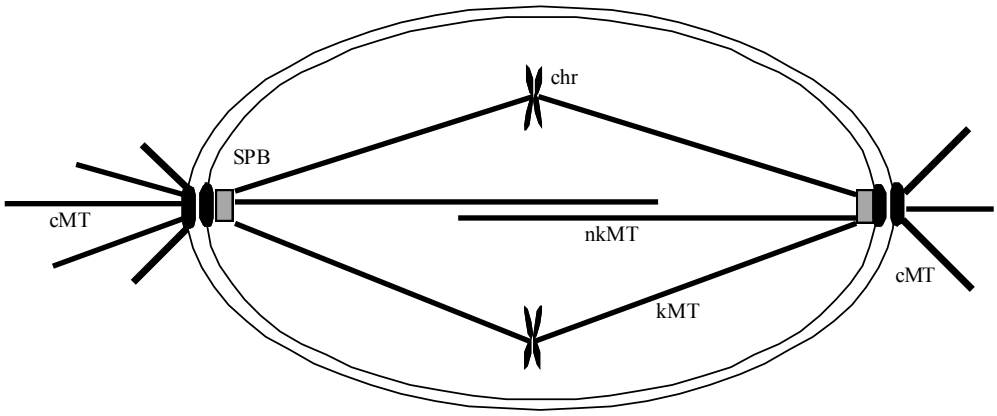


Figure 1.5 Microtubules of the yeast mitotic spindle
SPB: spindle pole body
Chr: chromosome
cMT: cytoplasmic microtubules
kMT: kinetochore microtubules
nkMT: nonkinetochore microtubules

Tub4p, the yeast γ-tubulin, nucleates MTs and is localized to the outer and inner plaques[21]. The minus ends of MTs proximal to the SPB appear to be static and capped, stably anchored to the SPB, whereas the majority of MT plus ends (which are oriented away from the SPB) are dynamic and splayed[22]. Morphologically, these structures correlate with stabilized (i.e. capped) and destabilized (i.e. splayed) MT ends[23].

The function of cytoplasmic microtubules is to promote nuclear migration during mating and orient the mitotic spindle at the site of cytokinesis during cell division[24, 25]. There are three to five cytoplasmic MTs per haploid cell[24]. Nuclear MTs are further subdivided to two classes. Kinetochore microtubules connect chromosomes to the SPB via kinetochores. They contribute to the alignment of sister chromatids on the metaphase plate and generate the tension needed to satisfy the spindle checkpoint[1]. Interpolar (non-kinetochore) MTs interconnect the two SPBs, forming an interdigitating array of

antiparallel MTs that provide physical linkage between the two halves of the spindle. The overlap region is termed the spindle midzone.

1.6 MICROTUBULE-ASSOCIATED MOTORS IN *S. CEREVISIAE*

Microtubule-based motor proteins couple ATP hydrolysis to the generation of force that generates movement. Two families of motor proteins are associated with microtubules: kinesins and dyneins. Most kinesins and kinesin-related proteins move toward the plus-end of microtubules (and are therefore considered anterograde motors) but some members exhibit minus-end directed motility[26]. Dynein is exclusively retrograde (i.e., exhibits minus-end directed motility)[27].

In vitro, kinesins and dyneins translocate microtubules at a speed on the order of microns per second[1]. In the context of mitosis, force generation often translates to MT sliding. This, along with the ability of some motors to act as microtubule cross-linking proteins, gives mechanochemical motor proteins the ability to regulate microtubule length and dynamics in the mitotic spindle.

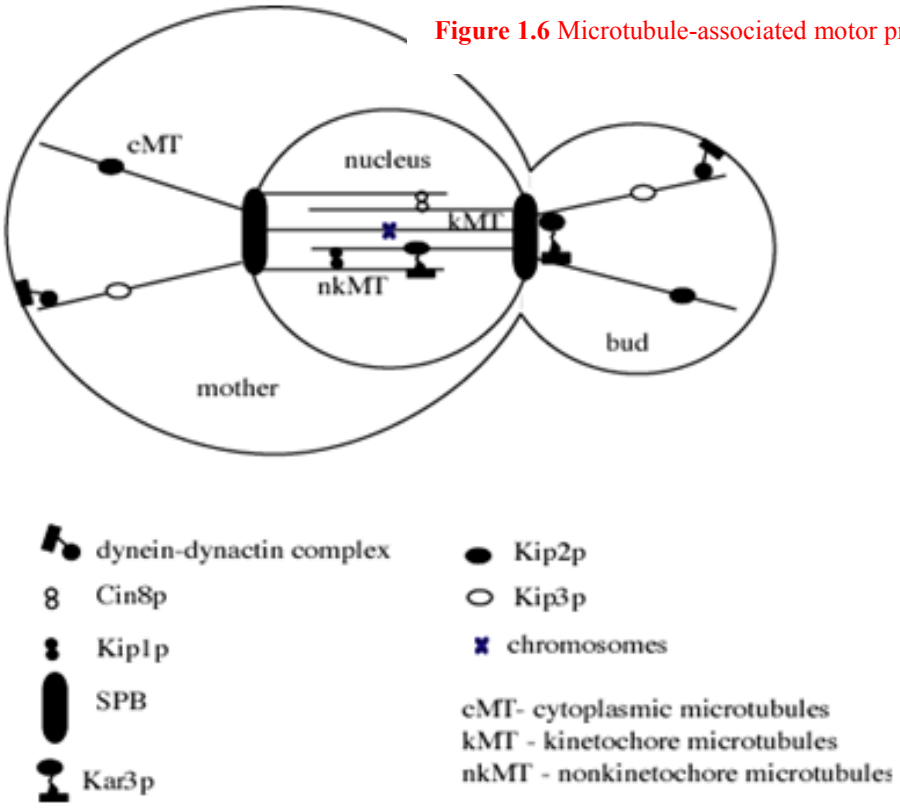
Seven MT-associated motors have been identified in *S. cerevisiae*[28] (see Figure 1.6). Six of them, Cin8p, Kip1p, Kip2p, Kip3p, Kar3p and Smy1p belong to the kinesin superfamily of motor proteins. The sequence similarity of these motors does not extend beyond the motor domain. The seventh motor, dynein, is a multisubunit complex that is best characterized by its heavy chain gene *DYN1*[29]. All of these motors function during mitosis except for Smy1p, which has a role in polarized cell growth[30].

CIN8 was isolated by its ability to complement mutations that result in chromosome loss[31]. A genetic screen for mutations that make *KIP1* essential for viability

identified *CIN8*[32]. Kip1p and Cin8p are both members of the BimC subfamily of kinesin-related motor proteins, whose members share a consensus sequence at the C-terminal. They have essential roles in spindle assembly and are required for the maintenance of spindle bipolarity until anaphase[33] (i.e. to prevent an inward collapse of separated poles). Specifically, prior to anaphase, the plus-end directed activity of these motors allows cross-linking and sliding of antiparallel polar microtubules of the spindle midzone, which is required for bipolar spindle formation. As the nuclear envelope does not break down during mitosis in yeast, a short intranuclear spindle assembles between the SPBs. The outwardly-directed force generated is also responsible for the spindle pole separating activity and elongation during anaphase[34] and is balanced by an inward-directed force acting upon the poles produced by Kar3p, a minus end-directed motor[32]. This antagonistic action is required for controlled spindle elongation.

Mutations in *DYN1*, *KIP2*, or *KIP3*, which interact primarily with cytoplasmic MTs, lead to failure to position the preanaphase spindle at the bud neck[35]. Misorientation of the anaphase spindle in *dyn1* or *kip3* mutants translates to increased frequency of spindle elongation within the mother cell, resulting in the generation of binucleate mothers and anucleate daughters. A similar nuclear migration and orientation defect arises in mutants lacking in cortical attachment components for astral MTs, such as dynactin, *kar9*, or *num1*[36]. In the case that the spindle becomes misaligned and the daughter nucleus is not successfully delivered to the bud following mitosis, a post-anaphase nuclear migration checkpoint introduces a compensatory delay of cytokinesis until one pole of the anaphase nucleus enters the bud with the help of astral MTs, which stochastically push the "daughter" spindle against the cortex[37, 38].

Figure 1.6 Microtubule-associated motor proteins



Due to the high degree of functional redundancy, none of the motors discussed thus far is singly essential for viability, yet the mitotic processes made possible by different combination of these motors are critical for viability. This will be demonstrated in the next section.

1.7 THE ROLE OF MICROTUBULES AND MOTORS IN THE YEAST CELL CYCLE

Progression through START, the G_1/S transition that constitutes the point of commitment to a new round of cell division, triggers the initiation of three distinct yet parallel pathways: DNA replication, bud emergence by actin-mediated polarized growth, and SPB duplication (see Figure 1.7). Cellular factors partition asymmetrically to

accommodate this transition. For example, dynein is distributed on MTs directed toward the bud[24], and Num1p and Kar9p associate specifically with the mother and daughter cortex, respectively[36]. While DNA is replicated, the duplicated SPBs separate and migrate in the nuclear envelope to opposite sides of the nucleus to form a bipolar spindle. This means that microtubules go from being nearly parallel to each other when the two SPBs are adjacent to each other, to antiparallel when the spindle assumes its metaphase/anaphase orientation. SPB migration appears to be MT-dependent as it is inhibited by MT-depolymerizing drugs that collapse the spindle[11].

Transition from S-phase to G_2/M is characterized by completion of DNA replication, formation of a 2-μm bipolar spindle, and attachment of sister chromatids to the mitotic spindle. Microtubule dynamics are important for this last process, as it requires a 'search-and-capture' mechanism at the plus ends to probe for the kinetochore, form proper attachments, and generate tension. Sister chromatids can actually become attached to the spindle prior to the completion of DNA synthesis due to the close proximity of centromeres to early firing origins of replication, leading to the suggestion that the S and G_2/M phases may partially overlap in *S. cerevisiae*[1].

Cytoplasmic microtubules, as well as the motors associated with them, also become relevant to mitosis during the G_2/M transition. Kip3p, by virtue of its interaction with cytoplasmic MTs, acts to correctly position the spindle near the bud neck (the aperture between the mother and the bud). Dynein and dynactin contribute to nuclear orientation as well by mediating a 'search-and-capture' mechanism that involves transient, dynamic interactions of MT ends with the bud cortex, ultimately contributing to spindle penetration of the neck[24]. The process involves pulling on cytoplasmic MTs in the

minus end direction by dynein (which is anchored to the bud cortex by dynactin), such that MTs slide laterally against the cortex. MT shrinkage (depolymerization) ensues, while the distal end is anchored to the cortex. Together, these events propel the daughter nucleus through the neck during anaphase[39]. Furthermore, such enhanced dynamics are also essential for nuclear migration during karyogamy[40].

Metaphase spindles of a haploid cell contain a single MT connected to each of the 16 chromosomes, and eight overlapping MTs which connect both poles[41]. Spindle microtubules at this point undergo fast turnover that is thought to help correct erroneous (i.e., syntelic) attachments that occur during bipolar chromosome alignment[42].

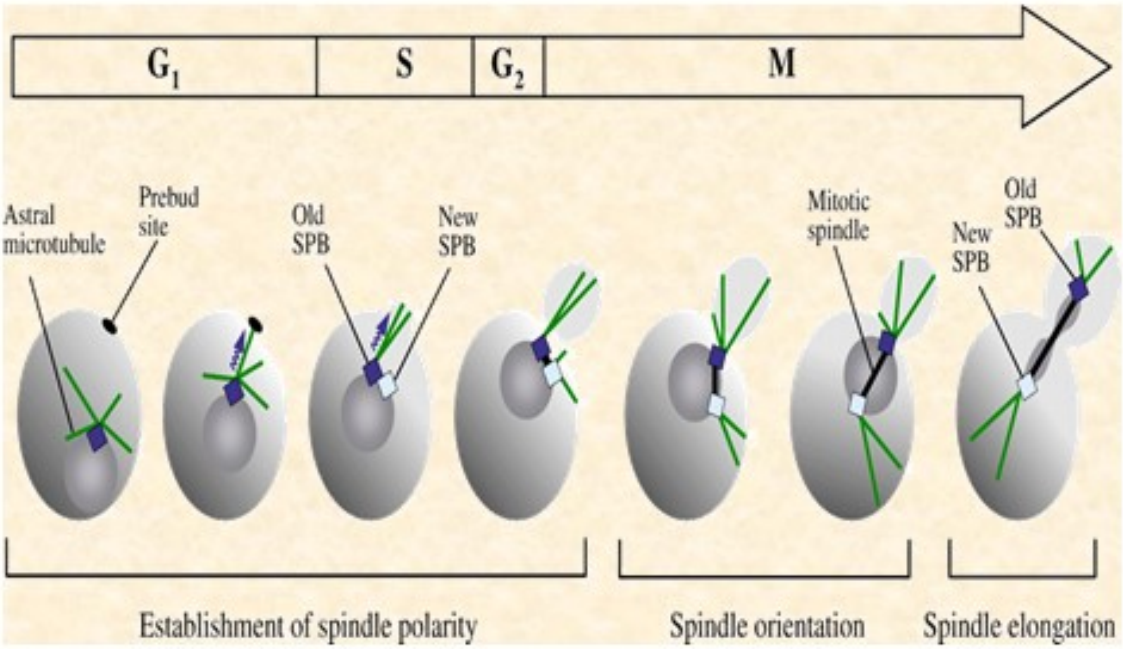
During anaphase A, chromosomes migrate toward the poles as kinetochore MTs shorten by depolymerization at kinetochores. In yeast, anaphase A happens concurrently or shortly after anaphase B[1]. During anaphase B, the spindle elongates as much as eightfold[43], penetrating into the bud. In late anaphase it reaches approximately 7-9 μm, spanning the mother-daughter axis (the distance from one end of the mother cell to the opposite end of the bud). The chromosome separating force is powered by Cin8p and Kip1p which cross-link and push apart interpolar 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. *cin8-3 kip1*Δ *dyn1*Δ triple mutants were shown to be incapable of anaphase B spindle elongation[34].

After proper chromosome segregation, the mitotic exit network (MEN) induces cytokinesis. The nucleus translocates into the bud separating the cytoplasm into two discrete compartments[44]. Bud scars containing chitin remain as rings on the surface of the

mother cell where dissolution of cell wall material occured, marking the position where the previous bud formed[45]

In summary, MT-dependent processes permeate practically every stage of mitosis (as well as some steps in preparation to it), and each stage in turn, is characterized by a specific MT architecture and dynamics (see Figure 1.7).

Figure 1.7 Spindle morphology during mitosis. From *Current Methods in Biology,1999*



1.8 CONTROL OF MICROTUBULE DYNAMICS AND STABILITY DURING MITOSIS

Forming a spindle was shown to involve a dramatic shift in dynamics, the half-life of MTs dropping from minutes to seconds owing to an increase in dynamic instability[46]. An increase in dynamic instability is essential when cells enter mitosis because elongation

and shortening events (like those discussed earlier) are crucial for spindle formation, capturing of kinetochores, and chromosome segregation.

In yeast, motors often considered as MAPs because they are potent regulators of MT dynamics. For instance, recombinant Kar3p fused with GST bound to glass slides was observed to depolymerize MT minus ends *in vitro*[47]. Furthermore, tis motor's overexpression was shown to cause spindle length to decrease[35]. Absence of dynein was shown to lead to a reduction of both MT growth and shrinkage rates, as well as the frequency of catastrophe, whereas the overexpression of *DYN1* was shown to result in increased length and bundling of cytoplasmic MTs in the bud[24]. Additionally, many of these cells exhibited nondynamic MTs, resulting in a decrease in the probability that MTs will depolymerize when their distal ends encounter the cell cortex[40].

Loss of Kar3p, Kip3p, or Dyn1p produced a phenotype of increased cytoplasmic MT number and length[48], while *kip2* mutant cells showed the opposite phenotype, with a reduction in the number[49] and length[35] of cytoplasmic MTs, suggesting a stabilizing role for this motor. Accordingly, elimination of Kip2p was shown to suppress the lethality and the long-MT phenotype produced by knocking out both *KAR3* and *DYN1* or *KIP3*. Furthermore, overexpression of *KIP2* was shown to produce extra-long cytoplasmic MTs[50]. These data suggest that Kip2p promotes Mt stabilization, while Kip3p destabilizes them.

Finally, mutant phenotypes of *kar3* and *kip3* were shown to be partially rescued by the MT-destabilizing drug benomyl[35] or by deletion of the nonessential α-tubulin gene *TUB3* which was demonstrated to decrease MT stability[48]. Expression of an extra copy of the *KAR3-1* allele, which compromises the motor function yet retains the ability to bind

MTs, was shown to partially stabilize MTs against depolarization by nocodazole[44]. Lastly, overexpression of *CIN8* was shown to result in the formation of abnormally long spindles[51].

Other than the contribution of individual MAPs and motors to MT stability, cell cycle mechanisms provide another level of regulation of mitotic processes by either modulating the levels or protein stability of these same MAPs and motors, altering their activity by post-translational modifications, or sequestering them to a specific cellular compartment. The key players that govern mitotic processes include the cyclins and Cdks, whose cyclin-dependent activity was shown to rise concomitantly with an increase in dynamic instability that occurs during mitosis[52]. Passage through START, for example, requires Cdc28p activation by the G_1 cyclins Cln1, 2, and 3[53], whereas progression through the S and G_2/M phases requires Cdc28p activation by the B-type cyclins Clb1-6. Cdc28p is the catalytic subunit of the main cell cycle cyclin-dependent kinase (CDK). Interestingly, strains containing *CLB* or *CDC28* deletions are unable to assemble a bipolar spindle due to a failure to segregate duplicated SPBs (which remain in a side-by-side configuration and arrest after the S phase with a 2N DNA content[54]). This phenotype is reminiscent of strains carrying *CIN8* and *KIP1* deletions. The authors proposed that the involvement of Cdc28 and its binding partners in SPB separation is direct, i.e., they either potentiate, via phosphorylation, Cin8p and Kip1p motors or impede Kar3p, the antagonistic motor. Evidence for this and more about the implication of cyclins in processes relevant for mitosis will be discussed in later chapters.

SUMMARY

Microtubules, microtubule-based motors, and MAPs together form the active, force-generating components of the spindle machine. Because processes that affect spindle stability and integrity must be tightly regulated, we employed specific genetic screens to identify additional regulators of spindle function. As a result of this study, proteins with a previously uncharacterized role in MT stability were found. These proteins may be candidate MAPs or novel regulators of MAPs (whether direct or indirect). In addition, a putative pathway to MT stability was drawn based on genetic interactions, epistatic experiments, and physical data, with additions to the model from the literature.

CHAPTER II

ISOLATION AND DOMAIN ANALYSIS OF A MULTICOPY SUPPRESSOR OF STRAINS COMPROMISED FOR MICROTUBULE STABILITY

Introduction

To study proteins that regulate microtubule stability we chose a system in which microtubule stability is deficient and mitosis is consequently compromised. The system was engineered by a deletion of the dynein heavy chain *DYN1* (whose protein product links cytoplasmic MTs to the cell cortex), as well as by introduction of a temperature-sensitive allele of *CIN8,* the motor responsible for cross-linking intranuclear MTs at the spindle midzone. The defect in microtubule stability of this background is evident upon examination of the rescuing agent: introduction of the *tub2-402* allele, which was previously shown to increases the resistance of cells to the MT-destabilizing drug benomyl[10], suppressed the temperature sensitivity associated with *cin8-3 dyn1*Δ cells[55]. This suggests that our background is deficient in MT stability.

Beyond the contribution of these motors to microtubule stability, the proteins play specific roles in mitotic processes. As discussed in Chapter 1, Dyn1p plays a key role in both spindle positioning and spindle elongation[24, 34], whereas Cin8p has a dominant role in bipolar spindle formation and spindle elongation[33]. Although individually the proteins are nonessential for these processes[32], they nonetheless cooperate on an essential process where they overlap in function, in this case, spindle elongation in anaphase B. Consequently, synthetic lethality arises in strains that contain a combination of *dyn1* and *cin8* deletions. To avoid complete lethality associated with complete loss of both motors, the *cin8-3, dyn1Δ* double mutant was generated which grows normally at 26ºC but ceases to grow at temperatures above 35ºC[34].

The temperature-sensitive *cin8-3* allele results from an amino acid substitution of Arg196 to Lys in the motor domain of Cin8p. This mutation affects the motor properties

of Cin8p but not the binding of the Cin8p to microtubules *in vitro*[56]. In fact, the authors showed that this microtubule-binding activity is more important for spindle formation and SPB separation than its motor function. Moreover, Crasta et al.[57] proposed that the bundling activity of Cin8 alone generates mechanical force of sufficient strength that catalyzes the conversion of interdigitating MTs emanating from 'side-by-side' SPBs into a parallel array, forming a short spindle.

We aimed to enhance the effect of a pathway or components of a pathway that regulate microtubule stability in cells deficient in MT-dependent processes. To do this, our lab previously screened a genomic library for gene products that, when present in excess, suppress the temperature sensitivity associated with *cin8-3 dyn1*Δ haploid cells[58]. We speculate that such gene overexpression would enhance a pathway that leads to MT stability.

The *cin8-3 dyn1*Δ cells form bipolar spindles at elevated temperatures but fail to promote spindle elongation in anaphase[34]. These cells were transformed with a yeast genomic library carried on the multicopy plasmid YEp24. The library was constructed by insertion of genomic fragments that average 10 Kb into the BamH1 cloning site of the plasmid[59]. The YEp24 vector is present in about 60-100 copies per cell[60], which translates to an approximately ten-fold overexpression of the inserted gene product[61].

One suppressor turned out to be an opposite *MAT* locus, which contains regulatory genes that determine mating-type and ploidy-depenent phenotypes, such as those that promote transcription of genes required for mating (in haploid cells) and sporulation (in diploids). It was hypothesized that co-expression from both *MAT* loci induced stabilization of MTs[62]. Another suppressor, clone #281, was partially sequenced

and localized to chromosome XIII. This chapter will discuss the isolation of the gene responsible for the suppression from this genomic insert, provide a characterization of the suppression, and test for potential mechanisms through which the suppressor may act.

Materials and Methods

Strains and Media

Strains used in the screen are listed in Table 2.1. All strains are either S288C derivatives or were backcrossed at least seven times to a S288C background. The *dyn1* deletion allele was obtained by transforming cells with a linear DNA fragment containing the *HIS3* gene flanked by *DYN1* sequences, designed to delete the *DYN1* ORF, leaving only 78 bp and 63 bp at the 5'- and 3'-end, respectively[29]. The *cin8-3* allele has been described previously[32].

Strain DEY2169 was constructed by crossing MAY2169 with YNN282 (Yeast Genetic Stock Center, University of California, Berkely) followed by tetrad dissection and analysis as described in Methods in Yeast Genetics[63]. In brief, diploids resulting from the mating were selected on double-dropout medium lacking leucine and tryptophan (SC-leu-trp) and were sporulated. Upon tetrad dissection by micromanipulation, haploid strains were selected that do not grow on medium lacking tryptophan, contain the *kip1::HIS3* deletion cassette and are temperature sensitive. Growth of DEY2169 at 36ºC was restored upon introduction of a centromeric plasmid carrying *CIN8*.

Media preparation and yeast genetic techniques were essentially as previously described[63]. Sensitivity to benomyl was tested on YPD-agar rich media to which the

desired amount of benomyl (Aldrich Chemical Company, Inc, Milwaukee, WI) was added from 10 mg/ml stock in DMSO.

Table 2.1 List of *Saccharomyces cerevisiae* strains and plasmids

Strain	Genotype	Source
MAY589	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101*	M.A.Hoyt
DEY589	*MAT***a** *dyn1Δ::HIS3 cin8-3 his3-Δ200 leu2-3,112 ura3-52 lys2-801*	D. Eshel
MAY2169	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101*	M.A.Hoyt
YNN282	*MAT***alpha** *his3-Δ200 ura3-52 ade2-101 lys2-801 trp1Δ*	YGSC*
DEY2169	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ*	This study
Plasmids		
YEp24	2μ *URA3*	NEB**
YEplac112	2μ *TRP1*	NEB**
pMA1125	*CIN8 URA3 CEN*	M.A. Hoyt
Clone #281	*FCP1 PGM3* 2μ *URA3***	D. Eshel
YEp24-*FCP1*	*FCP1* 2μ *URA3*	This study

YEplac112-*FCP1*	*FCP1* 2μ *TRP1*	This study
YEp24-*FCP1*(D182E)	*FCP1(D182E)* 2μ *URA3*	This study
YEp24-*FCP1*(D180E)	*FCP1(D180E)* 2μ *URA3*	This study
YEplac112-*FCP1*(ΔCT)	*FCP1* 2μ *TRP1****	This study
pN1835	*SSU72* 2μ *URA3*	S. Krishnamurthy
pKO100	*PTC2* 2μ *LEU2*	A. Kikuchi
pKO102	*PTC4* 2μ *LEU2*	A. Kikuchi
pKO117	*PTC1* 2μ *LEU2*	A. Kikuchi
pLG36	$P_{GAL>}$cin8-3-BCP *LYS2 CEN*	M.A. Hoyt

*Yeast Genetic Stock Center, University of California, Berkely

**New England Biolabs Inc., Beverly, MA, USA

***See text for details

Multicopy Suppressor Screen

The YEp24 based yeast genomic library (gift of M. Carlson, Columbia University, New York, NY) was prepared by cloning yeast genomic fragments (about 10kb average size) into the *Bam*HI site of the vector. Transformation of yeast with the genomic library was performed as described by Gietz et al.[64].

The genetic screen performed is as previously described[58]. In brief, after transformation of *cin8-3 dyn1Δ* cells with the library, colonies were picked which grew at both the permissive (26ºC) and the non-permissive (36ºC) temperatures upon replica-plating (see Figure 2.1). Plasmids were isolated from these colonies and were used to transform the original strain and test for suppression in order to confirm the results.

Plasmids carrying the genomic insert containing the suppressor were sequenced and compared to the *Saccharomyces* Genome Database (SGD.org) to determine the coordinates of the insert and the genes it contained.

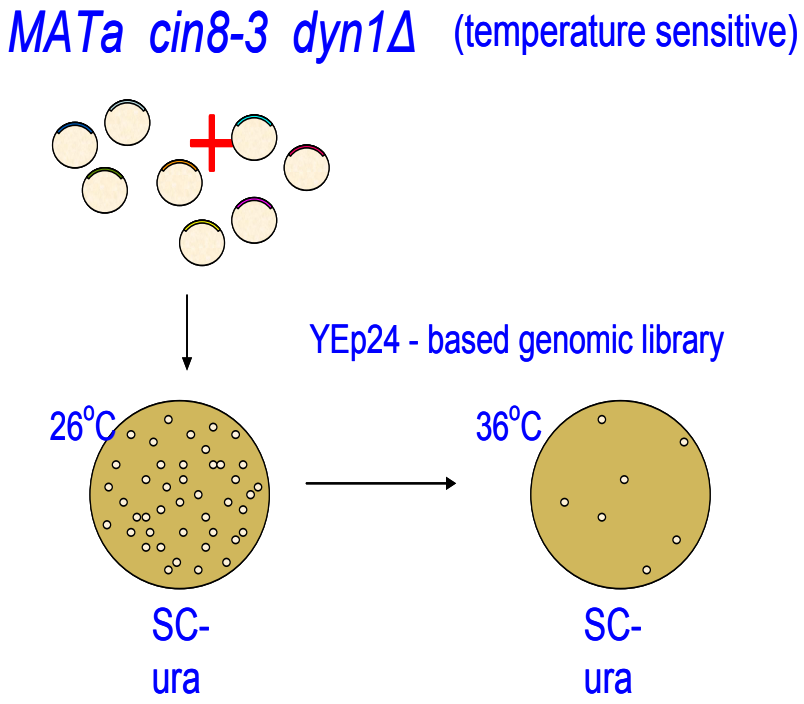


Figure 2.1 Design of theYEp24-based genomic library screen

Microscopy

To visualize DNA, liquid cultures of yeast cells were fixed by adding 2 volumes of cold 100% ethanol and incubated for 20 minutes on ice. Cells were washed twice with distilled water, incubated 30 minutes in the dark with 1 µg/ml 4',6-diamidino-2-phenylindol dihydrochloride (DAPI, Sigma Chemical Co., St. Louis, MO), washed twice with distilled water and mounted on a glass slide for observation. All samples were

viewed on Nikon Microphot-FXA or –SA microscopes equipped with epifluorescent optics and appropriate sets of filters using 60x oil-immersion objective.

Construction of multicopy plasmids containing FCP1

All enzymes used in cloning were purchased from New England Biolabs and used according to manufacturer's protocols. Clone #281 contained a 5466 bp fragment from chromosome XIII, spanning coordinates 819,738-825,204 inserted at the BamH1 site of the multicopy vector Yep24 (see Figure 2.2).

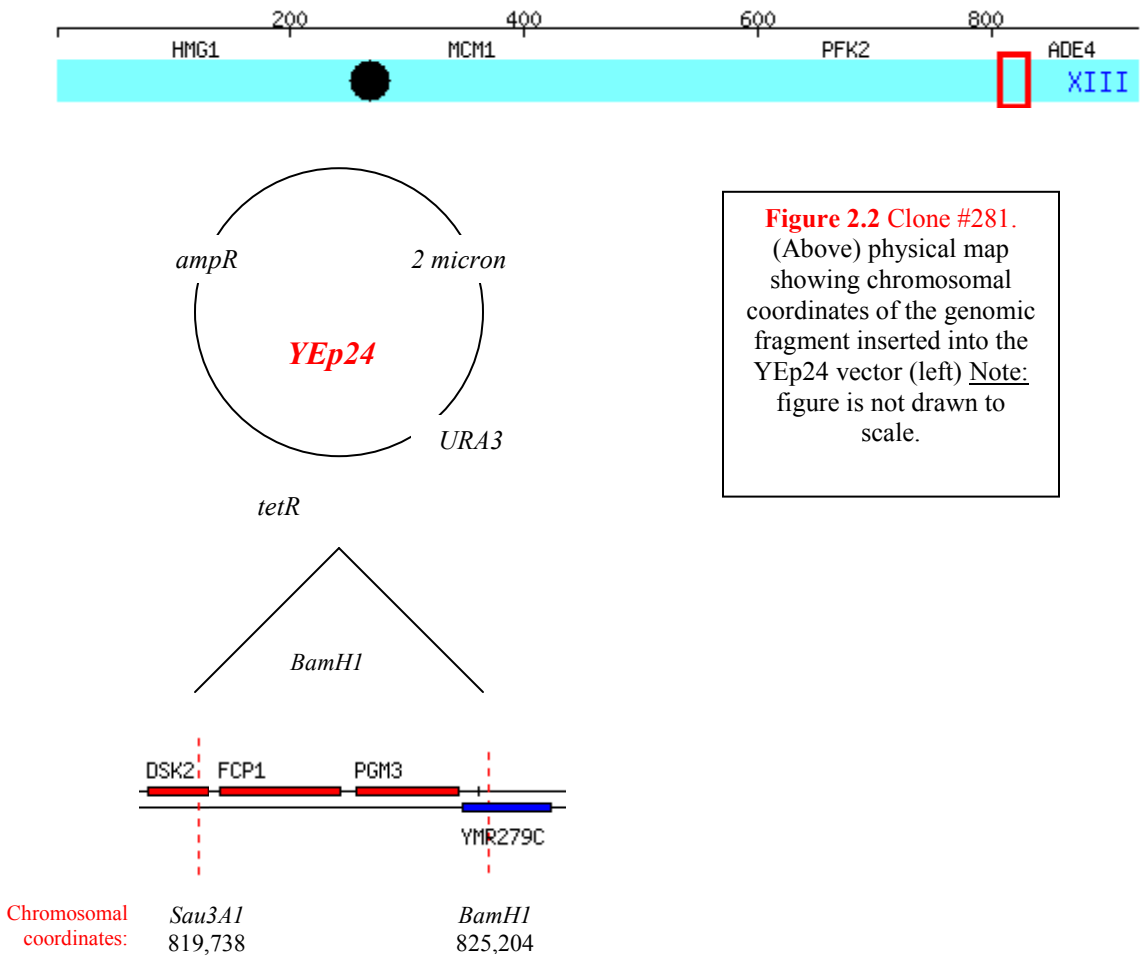


Figure 2.2 Clone #281. (Above) physical map showing chromosomal coordinates of the genomic fragment inserted into the YEp24 vector (left) Note: figure is not drawn to scale.

The genomic fragment contained two structural genes, *PGM3*, a gene coding for a glycolytic enzyme [65] and *FCP1*, an essential gene coding for a phosphatase in the general transcription machinery[66]. We decided to determine whether *FCP1* is implicated in the suppression. The plasmid was digested to isolate *FCP1* with XhoI (which cuts ~280 bp after the stop codon) and SmaI (that cuts the plasmid at a non-coding region), releasing a 2471 bp fragment containing *PGM3* (see Figure 2.3). The 10.76 Kb digested plasmid containing *FCP1* was gel purified using Qiaex II Gel Extraction Kit (Qiagen). Klenow fragment was used to fill in recessed 3' ends created by XhoI digestion to create blunt ends. Dephosphorylation of the 5' end of the linearized plasmid using Calf Intestinal Phosphatase (CIP) and blunt-end ligation using T4 DNA Ligase followed to allow circularization of the new plasmid with the SmaI-digested end. The plasmid was propagated in bacteria as previously described[67]. Restriction analysis confirmed the successful integration of the intact *FCP1* gene under control of its native promoter onto the YEp24-*FCP1* plasmid.

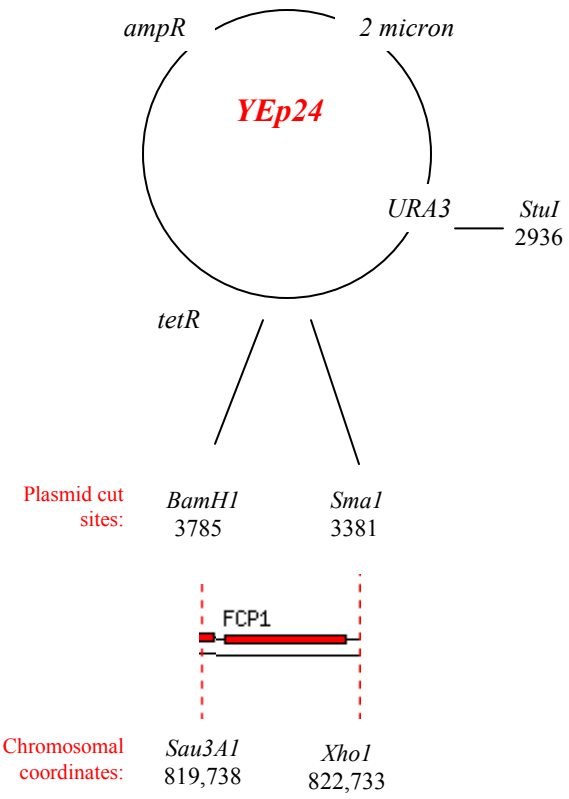


Figure 2.3 YEp24-*FCP1* plasmid.
Digestion of clone #281 (Figure 2.1) to isolate *FCP1* on the plasmid.
Note: figure is not drawn to scale.

FCP1 was also cloned onto another multicopy plasmid (YEplac112) containing a different selectable marker, *TRP1*. The *YEp24-FCP1* plasmid was digested with XbaI and StuI, effectively truncating the *URA3* gene (see Figure 2.4). In parallel, the YEplac112 plasmid, cut with XbaI and SmaI and dephosphorylated with CIP, was purified by size fractionation using Microcon Ultracel YM-50 Centrifugal Filter Device (Millipore) according to manufacturer's instructions. The 3.44 Kb piece containing *FCP1* was gel purified as before and integrated into the polycloning site of YEplac112. Blunt-end ligation, propagation in bacteria, blue-white screening, and confirmation by restriction analysis followed as before.

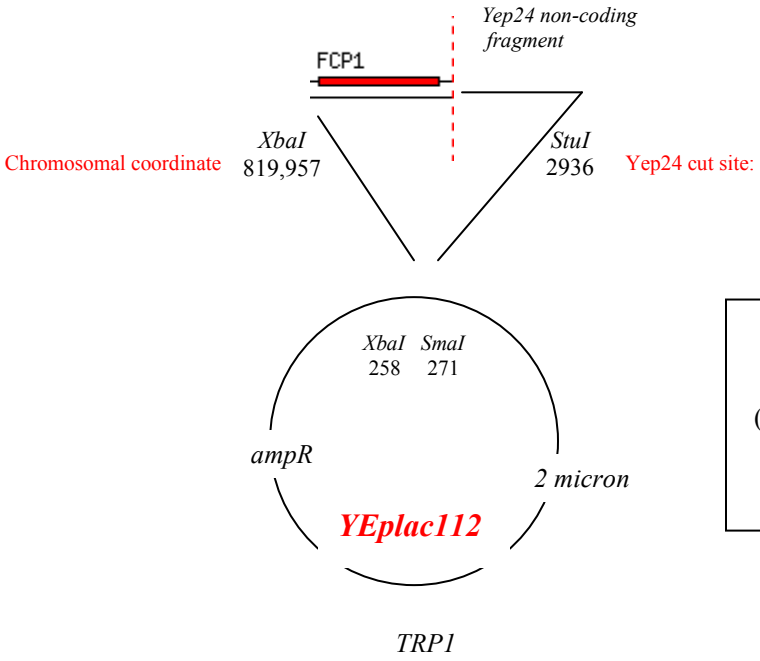


Figure 2.4 *YEplac112-FCP1* plasmid.
Digestion of YEp24-FCP1 (Figure 2.2) and its subcloning in YEplac112. Note: figure is not drawn to scale.

Generation of mutants of *FCP1*

Point mutations in the phosphatase domain of the *FCP1* gene on the YEp24-*FCP1* were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. The primers used to generate the D182E are 5'-TATCTTAGTGGTGGATTTAGAGCAAACCATCATTTGTG-3' and 5'-CACAATGGATGATGGTTTGCTCTAAATCCACCACTAAGATA-3' and the primers used to generate D180E are 5'-GAAACTTATCTTAGTGGTGGAGTTAGATCAAACCATCATCC-3' and 5'-GGATGATGGTTTGATCTAACTCCACCACTAAGATAAGTTTC-3'. Sequencing and BLAST were used to confirm the generation of the mutation.

To generate a plasmid containing a C-terminal deletion of *FCP1,* the YEplac112-*FCP1* plasmid was digested with BspEI and XbaI. The truncated *FCP1*, which spans genomic coordinates 819,957-821,835, was gel purified as before and subcloned into the

polycloning site of a new YEplac112 shuttle, which was digested with XbaI and BamHI. Klenow fragment, CIP, and blunt-end ligation were used as before to generate Yeplac112-*FCP1ΔCT*.

Western blot analysis

10-mL log phase cultures were spun down and resuspended in 1mL ice-cold TE buffer. Total protein lysate was prepared by vortexing cells with glass-beads in 50 µL Yeast Extraction Buffer (0.6% SDS, 10 mM Tris, pH 7.4 with addition of 1 µg/mL leupaptin (Sigma); 2 µg/mL aprotinin (Sigma); 1 µg/mL pepstatin (Sigma); and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). 50 µL Sample Buffer (125 mM Tris-HCL, 20% glycerol, 4.1% SDS, 4% β-mercaptoethanol) was then added, spun, and the supernatant was boiled for 5 minutes. 5 µL was mixed with an equal volume of Laemmli Sample Buffer (Bio-Rad) and loaded on 4-12% Gradient NuPAGE Novex Bis-Tris Mini Gel (Invitrogen), according to manufacturer's instructions. EZ-Run Pre-Stained Protein Marker (Fisher Scientific) was loaded as well. Western blot was performed as previously described[68] using Hoefer TE 22 tank transfer unit and Hybond ECL Nitrocellulose Membrane (Amersham Biosciences). Rat anti-tubulin antibody and peroxidase-linked goat anti-rat secondary antibodies were obtained from AbD Serotec. Mouse anti-3-phosphoglycerate kinase (PGK) antibody (Invitrogen) was a gift from P. Lipke and was used as a loading control in order to normalize the results. Peroxidase-linked anti-mouse IgG secondary antibody (GE Healthcare) was used at a 1:5000 dilution. To visualize the results, blots were developed using Amersham's enhanced chemiluminescence (ECL) Detection Reagents (GE Healthcare) in accordance with manufacturer's instructions and

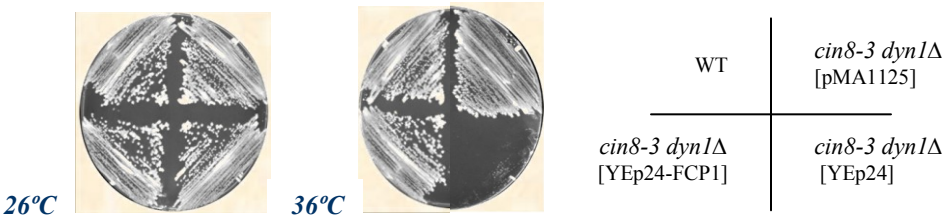
detected using Fujifilm LAS-4000. Fluorescence quantification and analysis was done using Fujifilm Multi Gauge software.

Results and Discussion

Fcp1 is a strong suppressor of the temperature sensitivity associated with *cin8-3 kip1*Δ and *cin8-3 dyn1*Δ cells

After isolating only *FCP1* on the plasmid, it was revealed as a "strong" suppressor of the temperature sensitivity of *cin8-3 dyn1*Δ mutants: it restored growth at the restrictive temperature (36ºC) to the same levels (i.e., colony size) as cells complemented with a wild type *CIN8* on a low-copy number plasmid (as compared to cells transformed with the control vector YEp24, see Figure 2.5). Introduction of YEp24-*FCP1* into a *cin8-3 kip1*Δ double mutant strain (which is also synthetically lethal) revealed that *FCP1* is strong suppressor in that background as well. More about this strain will be discussed in the next chapter.

Figure 2.5 Suppression of the temperature sensitivity of *cin8-3 dyn1*Δ

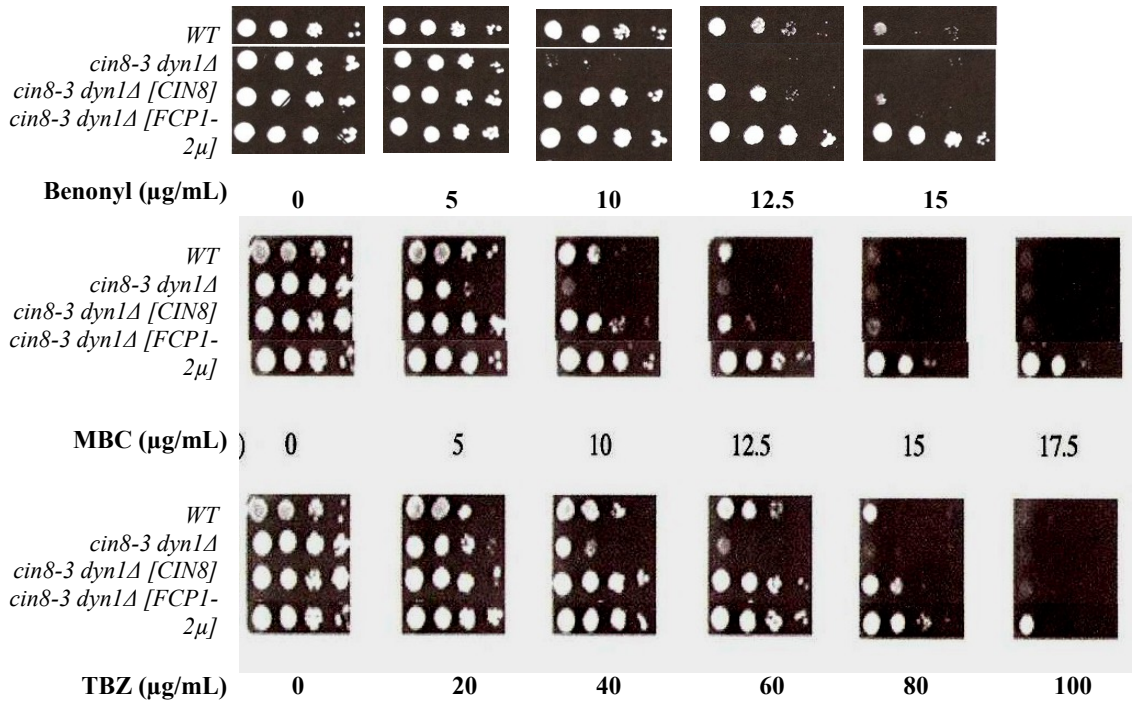


Fcp1 is a strong suppressor of the benomyl sensitivity associated with *cin8-3 kip1*Δ and *cin8-3 dyn1*Δ cells

Sensitivity to benomyl is considered to directly reflect MT stability MT[69]. The temperature sensitivity associated with *cin8-3 dyn1*Δ and *cin8-3 kip1*Δ may stem from the effects of microtubule destabilization, as these strains are sensitive to benomyl compared to wild type cells, and suppression of the phenotype is achieved by generating triple mutants of *cin8-3 dyn1*Δ *tub2-402* and *cin8-3 kip1*Δ *tub2-402* [55]. The *tub2-402* mutation increases the resistance of cells to benomyl, suggesting that this allele stabilizes MTs[10]. It is possible then, that the essential function compensated by our suppressors is not the motor activity of the mutated genes, but rather, the motors' putative role in MT stabilization.

FCP1 overexpression alleviates the sensitivity of the *cin8-3 dyn1*Δ double mutant to various microtubule-destabilizing drugs, including benomyl, thiabendazole (TBZ), and the more potent carbendazim (MBC), compared to the YEp24 control (see Figure 2.6). These drugs promote depolarization by sequestering tubulin dimers. The concentrations used in our studies were shown to reduce MT dynamics without substantially altering levels of polymer structures[70]. Furthermore, at high concentrations of these agents, suppression resulted in viability exceeding that of the *CIN8*-transformed mutant and of the isogenic wild-type strain MAY589 (see Figure 2.6).

Figure 2.6 Serial dilutions of various strains in the presence of different microtubule-destabilizing drugs



***FCP1* overexpression does not reduce the frequency of binucleated mothers in *cin8-3 dyn1*Δ cells**

In drawing a pathway to suppression of *cin8-3 dyn1*Δ temperature-sensitive mutant, one must consider the possibility that complementation of the function of either gene of the double mutant may result in modification of the stability and/or dynamics of nuclear MTs (which Cin8p interacts with) or of cytoplasmic MTs (which Dyn1p binds).

Stability of cytoplasmic MTs may manifest in alterations in nuclear migration[62] as this movement into the neck is powered by cytoplasmic MTs sliding along the bud cortex, which is mediated by dynein and dynactin [24]. In the absence of Dyn1p, when MT sliding is impaired, capture of the bud cortex by astral MTs ends followed by their shrinkage can serve as a backup mechanism to move the nucleus into the neck[71]. The

interactions that make up this mechanism are mediated by cortical anchorage components (i.e., Kar9p and Bim1p, see discussion in Chapter I). Still, nuclear migration is compromised in *dyn1Δ* mutants as these pathways only partially overlap and the cortical components mentioned do not facilitate sliding. Consequently, increased frequency of binucleate mothers and anucleate daughters is often observed in dynein/dynactin mutants. Because in these cells anaphase chromosome segregation is confined predominantly to the mother, we tested whether overexpression of *FCP1* suppresses this phenotype. DAPI staining of log phase cultures, however, revealed that the frequency of binucleate mothers in the *cin8-3 dyn1*Δ mutant was not altered by *FCP1* overexpression as compared to cells transformed with the control vector (not shown). This suggests that suppression of the temperature sensitivity of our strain by *FCP1* is not achieved through the correction of the nuclear orientation defect by either compensating for this function of dynein (i.e. bypassing its requirement in the process) or by reinforcing interactions of cytoplasmic MTs with cortical determinants at the bud site (i.e., enhancing search-and-capture or other correcting mechanisms).

***FCP1* overexpression does not affect tubulin levels in wild-type and mutant cells**

We next asked whether tubulin levels are altered in the double mutant relative to the wild-type and whether *FCP1* overexpression can affect tubulin levels, which may directly influence MT stability by increasing the pool of dimers available for polymerization. Since Fcp1 is a transcription factor in the general transcription machinery (see discussion in the next section) it is reasonable to hypothesize that it exerts its effect on MT stability by up-regulating Tub2p levels. Western blot analysis, however, demonstrated that Tub2p

levels in asynchronous populations is relatively similar in WT and double mutants and that protein levels are unaltered in cells overexpressing *FCP1* (see Figure 2.7). These results suggest that there may be another mechanism through which MT stability is mediated in cells bearing the *FCP1* multicopy plasmid.

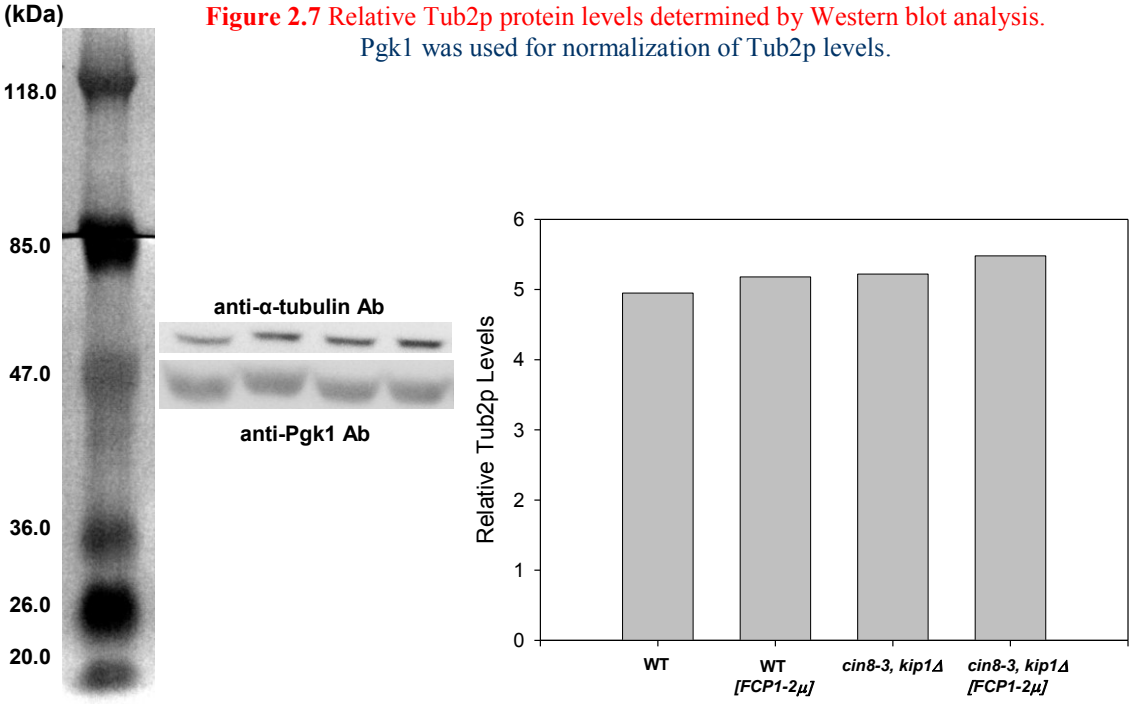


Figure 2.7 Relative Tub2p protein levels determined by Western blot analysis. Pgk1 was used for normalization of Tub2p levels.

Cin8p has been shown to bind and bundle microtubules in a rigor fashion in the absence of nucleotide or in the presence of AMP-PNP[56]. These conditions mimic the *cin8-3* allele that is altered at an arginine (R196K) that is conserved in the ATP-binding/hydrolysis domain of kinesin family members[72]. Studies revealed that the motility defect of this allele did not impair the ability of the motor to assemble the spindles and maintain their structural integrity, suggesting that the MT-binding activity is important for spindle assembly[56]. Overproduction of Cin8-3p, however, was excluded as

a possible mechanism for the suppression of the temperature sensitivity of our mutants as overexpression of *cin8-3* from a GAL-inducible promoter appeared to be toxic to the cells (i.e. it stunted growth at the permissive temperature; not shown).

The phosphatase activity of Fcp1p is required for suppression of the temperature sensitivity of *cin8-3 kip1*Δ and *cin8-3 dyn1*Δ mutants

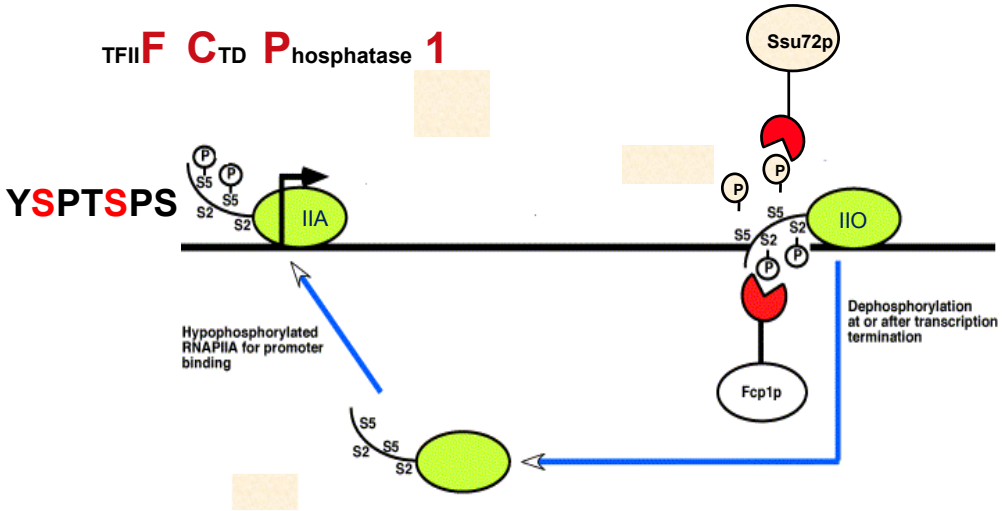
We decided to proceed with a different approach in our attempts to elucidate the mechanism of the suppression of the temperature-sensitivity of our mutants by *FCP1* overexpression. We hoped to accomplish this by determining the implication of functional domains of Fcp1 in the suppression. The rationale for this approach is that if a domain is shown to be involved in the suppression, then its associated function may contribute to an understanding of how the suppression is mediated.

Fcp1p (TFIIF-stimulated CTD Phosphatase 1) is a phosphatase in the general transcription machinery that specifically dephosphorylates Ser2-PO_4 of the tandem heptapeptide repeat Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 in the C-terminal domain (CTD) of the largest subunit (Rbp1) of RNA-polymerase II (RNAPII)[66]. In all eukaryotes, RNA polymerase II is distinguished from RNA polymerases I and III by a highly conserved C-terminal domain which forms a tail-like extension from the catalytic core of Pol II. It functions as a landing pad for myriad cellular factors that regulate the initiation, elongation, and termination steps of Pol II transcription, as well as RNA capping, splicing and polyadenylation[73]. The conserved heptapeptide (above) repeats 26 times in yeast and up to 52 times in metazoan CTDs[74] and it is the serines in this repeat

that are the target of phosphorylation and dephosphorylation events, or rather, waves, that the CTD undergoes during the transcription cycle.

The phosphorylation state of the CTD is generally believed to be the result of a balanced action of site-specific CTD kinases and phosphatases. In vivo, two forms of RNAPII coexist in dynamic equilibrium: the hypophosphorylated IIA form which assembles into preinitiation complexes, and the hyperphosphorylated IIO form (i.e., heavily phosphorylated at Ser-2 and Ser-5) that catalyzes transcript elongation and facilitates recruitment of the pre-mRNA processing machinery [75]. Upon transcriptional termination, CTD dephosphorylation catalyzed by Fcp1p is required to recycle the polymerase at the end of each round of transcription, an action that primes it for the next cycle[76] (see Figure 2.8).

Figure 2.8 The role of Fcp1p in priming RNAPII for another round of transcription

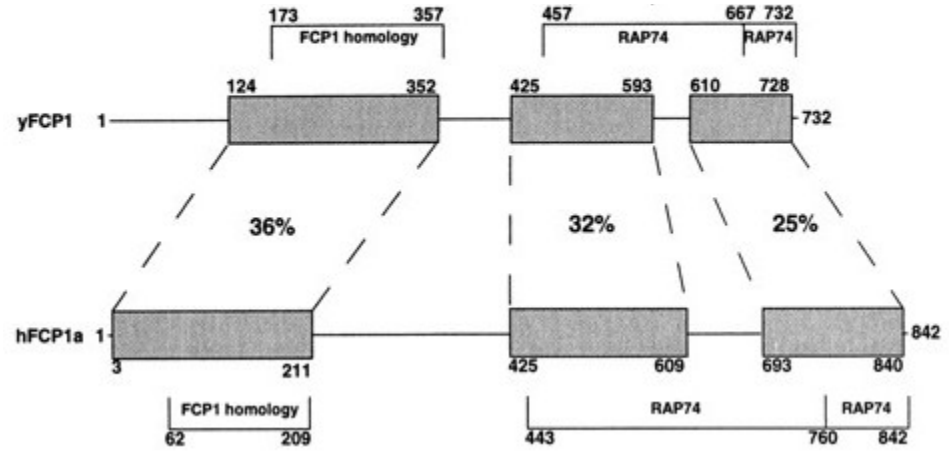


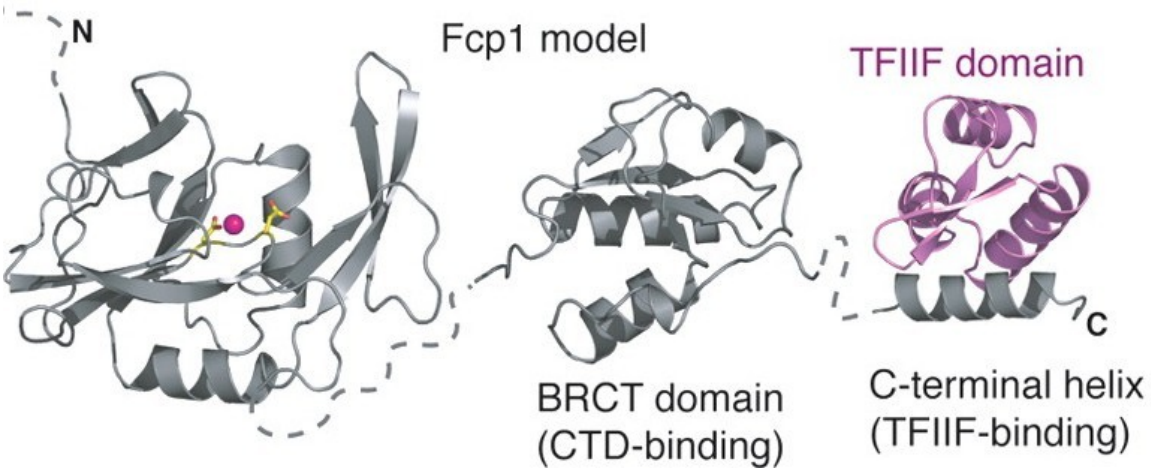
after Kobor & Greenblat, 2002

Initial characterization of Fcp1p revealed two conserved domains, an N-terminal FCP homology (FCPH) domain and a BRCT domain[77](see Figure 2.9). The FCPH domain consists of a ΨΨΨDXDX(T/V)ΨΨ signature motif at its catalytic center that was originally uncovered in a family of phosphotransferases and phosphohydrolases[78] (Ψ designates hydrophobic amino acids). The family is now known to include the putative tumor suppresosor HYA22[79] and the OS4 protein, which is frequently co-amplified in human sarcomas[80].

BRCT domains are commonly found in DNA damage-responsive cell cycle checkpoint and repair proteins, and are likely to mediate biologically important protein-protein interactions. Fcp1p is the only protein within the general transcription machinery that is known to have a BRCT domain[81]. Studies of the BRCT domain of Fcp1p were done in yeast and these revealed that a partial deletion of the domain is not able to complement a chromosomal *FCP1* deletion[66] and that the BRCT domain is required for Fcp1's catalytic activity *in vitro* and *in vivo*[82]. Crystallographic studies suggested that the integrity of the BRCT domain is essential for Fcp1p's function because it buttresses residues in the FCPH domain adjacent to the active site that are important for its catalytic activity[73]. Due to this intimate association with the FCPH domain, we could not disrupt the BRCT domain in order to account for its involvement in the suppression of the temperature and benomyl sensitivity of our strains.

Figure 2.9 Domain organization of Fcp1p. Regions of highest similarity and approximate locations of functional motifs in yeast and human Fcp1. Shaded areas are those most similar between yeast and human proteins. Rap74 is a subunit of TFIIF that binds to and regulates the activity of Fcp1. Domain demarcation taken from Archambault et al., 1997; domain structure taken from Meinhart et al., 2005



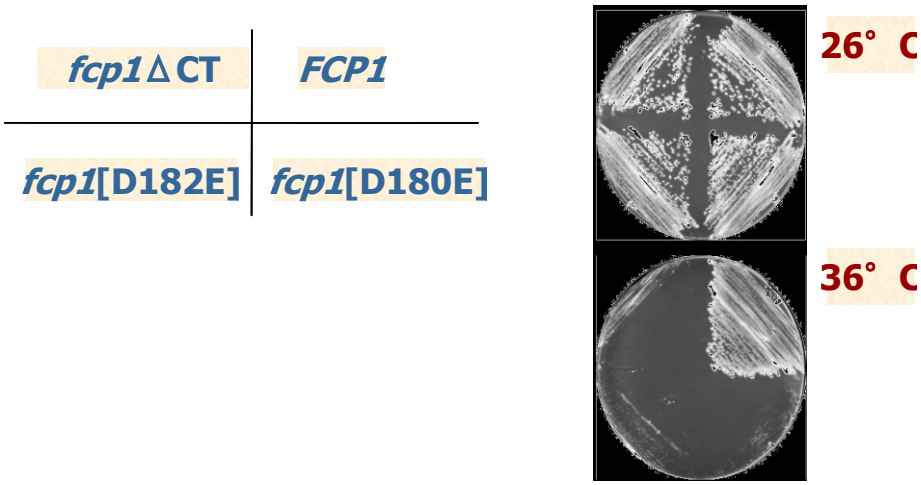


To determine whether the phosphatase activity of Fcp1p is necessary for the suppression of temperature sensitivity of our mutants, we separately generated amino-acid substitutions of the first and second conserved aspartatic acids of the ΨΨΨDXDX(T/V)ΨΨ motif. The first aspartate of the motif (D180) is the phosphoryl acceptor and the second (D182) contributes to metal ion binding and was suggested to act as a general acid/base[83]. Thus both aspartates are central to catalysis. Evidence for the

significance of these residues for catalysis was demonstrated by intolerance on the part of the enzyme for a conserved substitution: site-directed mutagenesis of Asp180 to Glu was shown to completely inactivate the enzyme, as the enzyme lost the ability to dephosphorylate the CTD of RNAPII *in vitro*. Furthermore, *in vivo*, mutations of neither aspartate could rescue the lethality associated with a chromosomal deletion of *FCP1,* even though the mutant proteins were shown to be expressed at similar levels to the wild-type protein[66].

Overexpression of *FCP1*(D180E) or *FCP1*(D182E) in our mutant strains could not suppress the temperature-sensitive phenotype (see Figure 2.10), suggesting that the integrity of the phosphatase domain is a prerequisite for suppression.

Figure 2.10 Overexpression of mutant alleles of *FCP1* abolishes the suppression of *cin8-3 dyn1Δ* cells



Since the phosphatase activity was shown to be essential for the suppression, we wanted to determine whether the effect of *FCP1* can be achieved by other candidate Ser/Thr phosphatases when overexpressed. It is conceivable that these candidate proteins

did not come up in our screen as expression of proteins in a genomic library screen may be masked by possible silencing effects of adjacent coding regions in the open reading frame or due to toxicity that results from overexpression of other gene products encoded by the same library clone. Since Fcp1p requires divalent cations and is resistant to phosphatase inhibitors such as okadaic acid and vanadate[84], we obtained multicopy plasmids carrying genes for phosphatases that have similar biochemical characteristics, namely, *PTC1*, *2*, and *4*[85]. None of these genes was able to suppress the temperature sensitivity of our strains when overexpressed (not shown). We also obtained a multicopy plasmid carrying *SSU72* from S. Krishnamurthy, which codes for a phosphatase specific for Ser5 of the same consensus sequence of the CTD of RNAPII[86] (see Figure 2.8). Ser5-PO_4 is specifically enriched in initiation and early elongation of transcription[73]. Overexpression of this functional analog, however, also did not suppress the temperature sensitivity of *cin8-3 dyn1Δ* cells (not shown).

The regulatory (TFIIF-stimulated) region of Fcp1p is required for suppression of the temperature sensitivity of *cin8-3 kip1*Δ and *cin8-3 dyn1*Δ mutants

A genome-wide expression profile demonstrated that expression of nearly 70% of all yeast genes is significantly reduced in an *fcp1* temperature-sensitive mutant[66]. Thus, *FCP1* is required for transcription *in vivo* and its inactivation leads to global defects in mRNA synthesis. Regulation of the phosphatase activity of Fcp1p involves an interplay of factors in the general transcription machinery: the enzyme's activity is stimulated approximately 10-fold by the RAP74 subunit of TFIIF, whereas TFIIB abrogates this stimulation[75]. The RAP74 binding site on Fcp1p is marked in Figure 2.9.

We sought to establish whether the phosphatase activity of Fcp1p that is necessary for the suppression of the temperature sensitivity of our mutants is independent of transcription or required only in that context. To achieve this goal we created a truncated version of *FCP1* that lacks the C-terminal region that was implicated in binding the RAP74 subunit of TFIIF and TFIIB. Unlike mutations or deletions to the BRCT domain which inactivate CTD phosphatase activity, a deletion of amino acids 626-732 of this C-terminal region only abolishes the protein's interaction with the RAP74 subunit of TFIIF and with TFIIB, yet maintains normal CTD phosphatase function *in vitro*[84]. Furthermore, in agreement with the activating role of TFIIF and TFIIB in transcription, the above deletion is not able to activate transcription *in vivo* from a LacZ reporter when artificially tethered to a promoter via a fusion to a heterologous DNA-binding domain[75]. Based on these observations, the authors suggest that this acidic C-terminal region could also act as a bona fide transcriptional activator.

Truncation of this region failed to suppress the temperature sensitivity of our mutants as well (see Figure 2.10), suggesting that the phosphatase activity that is essential for suppression of our mutants is dependent on functional targeting of Fcp1p to the transcriptional apparatus.

Conclusion

The basis of a multicopy suppressor analsysis is protein overproduction. Overproduction of many proteins, especially cytoskeletal proteins and their regulators, is toxic to cells[87-90]. Furthermore, overxpression may lead to mislocalization of certain proteins and to nonspecific interactions which may hinder interpretation of screen results. However,

despite these disadvantages, the significance of genes identified for MT stability outweighs this sacrifice. An advantage of overexpression analysis for our study is an opportunity to find genes that either complement or allow the compromised Cin8-3p to perform its functions, or alternatively, that modify Cin8p's substrate- microtubules. Those are some findings that cannot be identified by mutational analysis.

The results in this chapter suggest that the effect of *FCP1* overexpression may not be direct (i.e., mediated through physical interaction with components of the cytoskeleton) but rather may be due to the protein's effect on global gene transcription, as both the phosphatase activity and functional targeting to the transcription machinery was shown to be required for the suppression. The next chapter will describe a genetic screen designed to identify components that are either downstream of a putative '*FCP1* pathway' or that are necessary for *FCP1*'s effect on microtubule stability.

CHAPTER III

IDENTIFICATION OF GENES WHOSE DELETION BLOCKS THE EFFECT OF *FCP1* OVEREXPRESSION ON MICROTUBULE STABILITY AND THEIR PUTATIVE PATHWAYS

Introduction

To identify genes that are either downstream of a putative '*FCP1* pathway' or that are necessary for this gene's effect on MT stability, we chose to use the *cin8-3 kip1Δ* genetic background as opposed to *cin8-3 dyn1Δ* cells, as suppression of the temperature and benomyl-sensitivity was similarly established in both strains (see Chapter II). The Cin8 and Kip1 motors crosslink and slide spindle microtubules, an action that is required for mechanical separation of the duplicated SPBs and for spindle elongation in anaphase B[34]. At anaphase, specifically, Cin8p was shown to primarily promote the rapid phase of anaphase, whereas Kip1p was shown to promote the slow phase of anaphase spindle elongation[91].

Evidence for a defect in MT stability in *cin8-3 kip1Δ* cells comes from demonstrations that the *tub2-402* allele, which was shown to be benomyl-resistant and suggested to produce hyperstable MTs[48], suppressed the temperature sensitivity associated with *cin8-3 kip1*Δ cells[55]. Additionally, loss of or mutations in *KAR3* (an established microtubule destabilizing motor) in the *cin8-3 kip1Δ* background was shown to restore the ability of the double mutant to assemble the spindle and complete mitosis at the nonpermissive temperature[33, 92], while overexpression of *KAR3* in the same background was shown to have the opposite effect, namely, exacerbate the temperature sensitivity and produce a subsequent pre-anaphase arrest[51].

We chose to focus on *cin8-3 kip1Δ* cells for two main reasons. First, to specifically focus on spindle integrity that is solely dependent on MT stability, as opposed to spindle positioning which can be compensated by actin-dependent mechanisms (reviewed in Chapter I). *cin8-3 dyn1Δ* cells have a binucleated mother

phenotype that occurs in 23 % of cells[58], resulting in spindle alignment defects inherent in dynein mutants[29, 93]. This percentage did not waver significantly in cells overexpressing *FCP1* (see Results section in Chapter II), ruling out the possibility that the mechanism of suppression is mediated through repair of spindle alignment. We therefore wanted, in a secondary screen, to exclude genes that may correct this phenotype by microtubule-independent mechanisms and focus on genes more relevant to bipolar spindle formation and maintenance and to anaphase spindle elongation. These processes are a hallmark of *cin8-3 kip1*Δ cells.

The second rationale for employing the *cin8-3 kip1*Δ strain for our further studies is that when synchronized by α-factor at G_1, these cells become arrested at the G_2/M transition when released at the restrictive temperature due to inability to separate the SPBs and subsequently develop the mitotic spindle[32, 94]. On the contrary, *cin8-3 dyn1*Δ cells become arrested at various stages of the cell cycle[55]. Because of the lack of specificity in the timing of arrest in *cin8-3 dyn1*Δ cells, *cin8-3 kip1*Δ cells became the chosen background for identifying genes that are downstream of *FCP1* or that are required for its effect on microtubule stability.

Our secondary screen employed a Tn7-derived insertional genomic library of *S. cerevisiae*, generously donated by Dr. Anuj Kumar. This library was constructed using a Tn7-derived transposon that was used to mutagenize a plasmid-based library of yeast genomic DNA *in vitro*. Once transformed into the desired background, the plasmids containing Tn7 insertions can be used to create genomic truncations (see details in the Materials and Methods section). We used this insertional mutagenesis library to identify

genes downstream of or necessary for *FCP1*'s suppression of the temperature sensitivity associated with *cin8-3 kip1*Δ, as truncation of these genes will abrogate the suppression.

Materials and Methods

Strains and Media

Strains used in the screen are listed in Table 3.1. Media preparation and yeast genetic techniques were essentially as previously described[63]. Sensitivity to benomyl was tested on YPD-agar rich media to which the desired amount of benomyl (Aldrich Chemical Company, Inc, Milwaukee, WI) was added from 10 mg/ml stock in DMSO. Sensitivity to thiabendazole (TBZ, MP Biomedicals Inc.) was similarly tested and added from 10 mg/mL stock in DMF.

Table 3.1 List of *Saccharomyces cerevisiae* strains and plasmids

Strain	Genotype	Source
MAY589	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101*	M.A.Hoyt
DEY2169	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ*	This study
BBIL	*MAT***α** *mrs6::mrs6-2-LEU2 his3-Δ200 leu2-3,112 ura3-52 ade8 trp1Δ*	A. Ragnini
DEY3001	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ swi6::KanMX4*	This study

DEY3002	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ swi4::KanMX4*	This study
DEY3003	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ mbp1::KanMX4*	This study
DEY3004	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ whi5::KanMX4*	This study
DEY3005	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cln1::KanMX4*	This study
DEY3006	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cln2::KanMX4*	This study
DEY3007	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ cln3::KanMX4*	This study
DEY3008	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ clb1::KanMX4*	This study
DEY3009	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ clb2::KanMX4*	This study
DEY3010	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ clb3::KanMX4*	This study
DEY3011	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ clb4::KanMX4*	This study
DEY3012	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ clb5::KanMX4*	This study
DEY3013	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study

	ura3-52 ade2-101 trp1Δ clb6::KanMX4	
DEY3014	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ mih1::KanMX4	
DEY3015	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ swe1::KanMX4	
DEY3016	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ msn5::KanMX4	
DEY3017	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ slt2::KanMX4	
DEY3018	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ vps21::KanMX4	
DEY3019	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ bim1::KanMX4	
DEY3020	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ gin4::KanMX4	
DEY3021	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ hsl1::KanMX4	
DEY3022	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ elm1::KanMX4	
DEY3023	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ ats1::KanMX4	
DEY3024	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study
	ura3-52 ade2-101 trp1Δ boi2::KanMX4	

DEY3025	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ bud3::KanMX4*	This study
DEY3026	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ bni1::KanMX4*	This study
DEY3027	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ bnr1::KanMX4*	This study
DEY3028	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ smy1::KanMX4*	This study
DEY3029	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ kar9::KanMX4*	This study
DEY3030	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ bud6::KanMX4*	This study
DEY3031	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ bem3::KanMX4*	This study
DEY3032	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ bni4::KanMX4*	This study
DEY3033	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ kcc4::KanMX4*	This study
DEY3034	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ nap1::KanMX4*	This study
DEY3035	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ mrs6::mrs6-2-LEU2*	This study
DEY300	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101*	This study

	swi6::KanMX4	
DEY301	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101 swi4::KanMX4*	This study
DEY302	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101 clb2::KanMX4*	This study
DEY303	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101 hsl1::KanMX4*	This study
DEY304	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101 elm1::KanMX4*	This study

Plasmids

YEp24	2μ *URA3*	NEB**
YEplac112	2μ *TRP1*	NEB**
pMA1208	*CIN8 LEU2 CEN*	M.A. Hoyt
YEplac112-FCP1	*FCP1* 2μ *TRP1*	This study
YCplac33M6PN	*MRS6 URA3 CEN*	A. Ragnini

*Yeast Genetic Stock Center, University of California, Berkely

**New England Biolabs Inc., Beverly, MA, USA

***See text for details

Screening with the Tn7-insertional library

The insertional library was described by Kumar et al.[95]. In brief, the transposon used is bounded by Tn7 end sequences that act as substrates and binding sites for recombination proteins which mediate Tn7 transposition. To enable selection in *E. coli* and yeast, this

Tn7 mini-transposon carries the *tet* and *URA3* genes, respectively. The transposon also carries a promoter-less and 5'-truncated *lacZ* gene that serves as both a reporter and a simple gene trap for protein-coding sequence. The *lacZ* reporter is terminated by a series of stop codons that create a truncation of the mutagenized gene at the insertion site (see Figure 3.1).

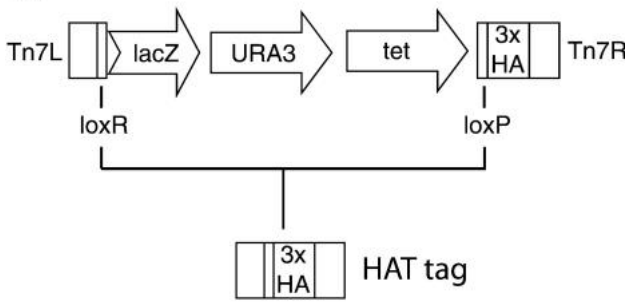
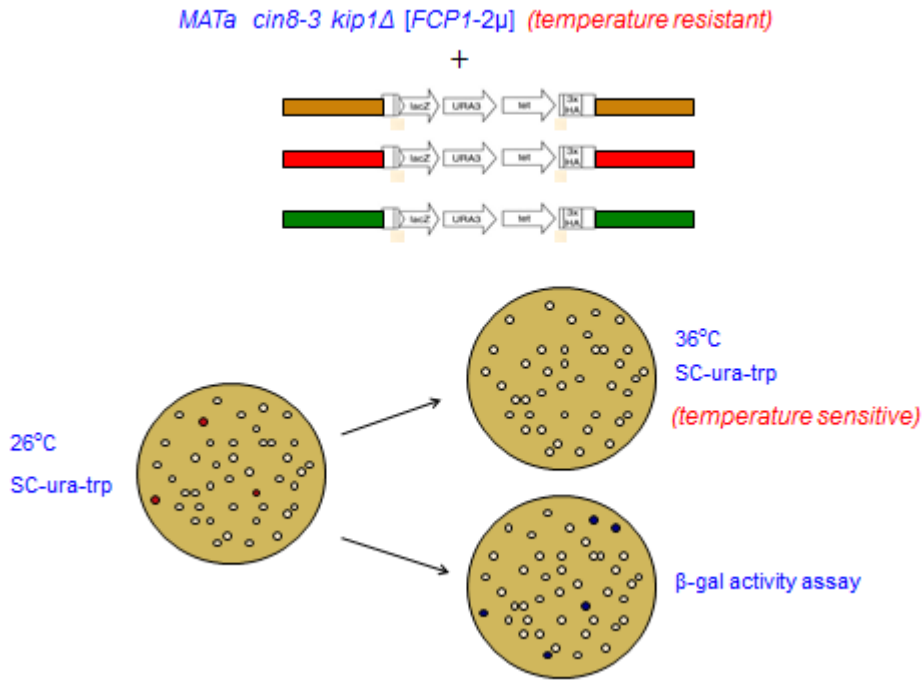


Figure 3.1 Tn7-derived mini-transposon used to mutagenize a yeast genomic library by insertion. Adapted from Kumar et al., 2004.

The library pools obtained from Dr. Kumar were first amplified in bacterial strain BL21 (DE3) as described in http://www.lsi.umich.edu/facultyresearch/labs/kumar/protocols. *cin8-3 kip1*Δ double mutants carrying a YEplac112-*FCP1* plasmid were transformed with a *NotI*-digested Tn7-derived insertional library according to the author's protocols. Gene disruptions were generated by homologous recombination as the mutagenized genomic DNA fragment replaced its native chromosomal locus. In our *cin8-3 kip1*Δ background, such transformations generated triple mutants. Transformants were allowed to grow at 26°C on large (30 cm) synthetic complete plates lacking uracil and tryptophan (SC-uracil-tryptophan), at a density guaranteeing resolution of individual colonies. Transformants were replica plated on the same media and incubated for 3 days at the nonpermissive

temperature, 36°C. Since all cells originally grew at the nonpermissive temperature (due to *FCP1* overexpression), colonies that ameliorate this effect were selected; i.e. those that grow at 26°C but not 36°C subsequent to insertional mutagenesis (see Figure 3.2).

Figure 3.2 Design of the screen for genes whose disruption ameliorates the effect of *FCP1* overexpression. Colonies selected for further studies where those that no longer grew at the nonpermissive temperature and gave a positive β-galactosidase activity test.

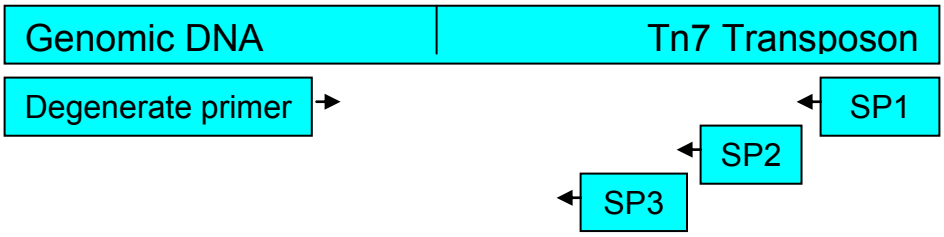


After confirming that the colonies indeed lost the ability to grow at 36°C by streaking them at the permissive and restrictive temperatures, in-frame integrations of *lacZ* fusions were tested by β-galactosidase activity screening (referenced above).

TAIL PCR and TA-Cloning

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[63]. Thermal Asymmetric Interlaced (TAIL) PCR was used to amplify flanking sequences adjacent to the insertions. The procedure was adapted from Gonzalez-Ballester et al.[96]. In brief, primary TAIL-PCR reactions used the degenerate primer Eco721 (containing an EcoRI site at the 3' end, in bold type: 5'-CCAGTGAGCAGAGTGACGIIIIINNSWC**CACGTG**-3', base notation: W, A+T; S, G+C; N, any nucleotide; I, inosine) and a Tn7-specific primer SP1 (5'-GTGCGGGCCTCTTCGCTATT-3'). Primary reactions were diluted 10-fold and 2.5-μL aliquots were added directly to secondary TAIL-PCR reactions (20-μL), which contained identical components and concentrations to the primary reaction except that the degenerate primer was replaced by primer Q0, which contains only the 5' sequences of primer Eco721 (5'-CCAGTGAGCAGAGTGACG-3') and the Tn7-specific primer was replaced by a nested primer SP2 (5'-ACGTTGTAAAACGACGGG-3'). For tertiary reactions, the secondary reactions were diluted as before and amplified with primer Q0 and with the doubly-nested primer SP3 (5'-GGCCTTCTTGCTTTGGAAGTAC -3', see Figure 3.3). In some cases a quaternary reaction was used with the primer SP4 (5'-GATCTTTTTTGTCCAGTTTGAG-3'). Amplified products from the reactions were analyzed by agarose gel electrophoresis, observing the development of a single prominent band.

Figure 3.3 TAIL-PCR scheme. SP: Tn7-Specific Primer



3-µL of fresh PCR product from tertiary PCR was ligated into vector pCR2.1 (Invitrogen) in a TA-cloning reaction, according to manufacturer's instructions. Amplification in *E. coli* and blue-white screening followed. Purified plasmids were digested with EcoRI to confirm the integration of the PCR product, and PCR was used to identify plasmids containing transposon sequences using Tn7-specific primers SP3 or SP4 (above) and (5'-GGCGGACAAAAAAGTCTC-3'). The plasmids containing Tn7 sequences were sequenced using pCR2.1 vector-specific primers M13 Reverse (-29) Primer (5'-CAGGAAACAGCTATGACC-3') and T7 Primer (5'-TAATACGACTCACTATAGGG-3'). 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.

Southern Blot

Purified chromosomal DNA from designated colonies was digested with HindIII overnight and separated through 0.8% agarose gel. DNA was blotted overnight onto nylon membrane NytranN (Schleicher & Schuell) using the Turboblotter Rapid Downward Transfer System (Schleicher & Schuell Inc., Keen, NH, USA) according to manufacturer's instructions. The blot was probed with a biotinylated probe corresponding

to the 3.25-kbp HindII fragment of the *lacZ* gene. This piece was gel purified using Qiaex II Gel Extraction Kit (Qiagen) and Random Primer Biotin labeling of DNA was performed using NEBlot Phototope Kit (New England Biolabs) according to manufacturer's instructions. NEB's Phototope Star Detection Kit was used and the membrane was exposed to an x-ray film.

Strain Construction

Strains from the *Saccharomyces* Deletion Project were a gift from Dr. Peter Lipke. Strain BB1L containing the *mrs6-2* allele is a gift from Dr. Antonella Ragnini. Deletion strains were propagated on YPD plates containing 200 ug/mL geneticin (Gibco, Invitrogen). Strain BB1L was propagated on SC-leu plates. DNA was isolated from these strains as above and the genes containing the KanMX4 or LEU2 cassettes were amplified using Finnzymes Phusion Hot Start High Fidelity DNA Polymerase (NEB) according to manufacturer's instructions. Table 3.2 lists the primers used to amplify the specific deletions from the yeast deletion strain collection. PCR products containing the deleted cassettes were verified by agarose gel electrophoresis and transformed, according to Davis Lab protocols http://depts.washington.edu/yeastrc/pages/fm_home3.html , into haploid *cin8-3 kip1*Δ double mutants carrying the 2μ plasmid YEplac112-*FCP1*. Geneticin-resistant colonies or leucine prototrophs were re-streaked on the same plates. DNA was subsequently purified from triple mutants and a confirmation primer (a few hundred base pairs upstream or downstream of the deletion) together with one of the original primers (see Table 3.2) was used to confirm the deletion cassette was integrated into the correct locus.

Table 3.2 Primers used to amplify the deletion cassettes from the yeast deletion strains

GENE	Forward primer used to amplify the gene	primer-specific coordinates	Reverse primer used to amplify the gene	primer-specific coordinates	WT size	deletion size	Confirmation Primer	
CLB6	ACACAGGACTTGGTTGTTTCTACTC	706800	ACACAGAAAACCAAAATCTCACTTC	705068	1735	2176	TTTGGTGAACTGGATAATCAAGATTC	R
MSN5	CCTATCTTTATTCGTTGAGCCTGTA	1140848	TGTATGCACGTACCTCTTACACAGT	1145119	4274	2183	CGAAAATGCTTCAGGTGGAGATAAAC	F
SWI4	tgtcactacaaattattg	386478	Gaaacgagaagaaggatc	382177	4301	2603	GTCTGAGAGAACAAAGCCAA	R
SWI6	ttgcgaccgatcttcttcact	517544	Gattttgagcgacgcgaaat	520758	3214	2386	TCATGTTGGATTTTAGGCAGG	R
MBP1	atagtatcgctggcgtttg	352134	Ttggaaggtgtcactgtcttg	355799	3665	2837	ATATCACTGGACGTGGTTGTG	F
WHI5	TTGTGCTGTTTATGATGTCTTTTGT	479215	GCAAATAGTACTCCCATACCACCTT	480805	1593	2289	CACTGCTACCGCTGATCACCTTAG	F
CLN3	GAGTCCCAGTCGCAATCTCACATC	67686	ACGATAGTAATCGTATTTTAGGTTGTGC	65578	2112	1914	GAGGAAACAAGAGATAATTGGTCATC	R
CLB2	GAATTGATACTTCTGAAAAGCGAAA	771308	TACTGATGCTTTTCTTTGTCTACCC	773472	2167	2275	GTGCCTTAGGGGGACTCAAACTTAA	F
SLT2	GCGGCGATTTTTATATTATCTTTTT	170645	TAAAGGGCTTCTCAGTGAATACATC	168500	2149	2278	CAAGATTGTGGATGACGAAGTTGTC	F
VPS21	CTACTTCAATAGTTATGAACCCGGA	491180	CAATATTAACTAATCCATCTCCCGA	489853	1330	2281	ATGACACCTATCTTTCGTATTCTTCC	R
BIM1	GACGCCTTGTATCTTCTATAGGTCA	187979	AGCTTGTACAGAATTGCTAACCAAA	189706	1730	2279	TCAGCAAAGGTAAACCTTTCTCATC	F
GIN4	CACACCATAGATTCCTTTTGATGTT	1466011	GAGGGCAAATAGCTTTTAAATCTTC	1462086	3928	2083	AAGAAACGGACCATACTGG	F
HSL1	TTGGATCGTAAGTTCGCTAGTTATC	248277	CTTATTCGACCTTCATCCTATCAGA	253519	5245	2272	TGCTGTCAGTATGAATATGGC	R
ELM1	TTTGGCTTTATAGTTGGTTCTTCAC	349144	CGATGAAGTTTTGTTTTTCTTTTGT	346521	2626	2287	AATGCCGTCTTTCTTTGGG	F
CLB1	TTCTAGGCAATTTTGGTTGTATTGT	703380	CATGGTTTATTTCAAGGTTTTTGAC	705384	2031	2199	AGGACACGATCGAGATTCCC	F
ATS1	TTTAAGTTACGTGTTGCATTGCGAGA	114798	CTTATGCTGTGCCCTTGTGCTT	113412	1390	1933	AGCATAAATGTTGGGTTGCC	R
BOI2	GAGGACAAATTCGTCAAGAAAGTAA	394029	GTCACTAAACAGATTAACGGCATCT	390357	3675	2136	AGGAAGAAGAAAACCCACG	F
BUD3	GGACAAAGATAATAAACCGCCTACT	95907	TTACTCGAGCTGTTAATGTTCTCCT	101118	3715	2314	GCAAAGTGCAAACCGTATCA	R
CLB3	CAGGAAGAATGAAAACATCTCAGTT	176476	ATTTGGTGGCAGTATATGAATGAGT	178329	1856	2156	GGTCACTAAAAATGATGGCG	R
CLB4	GTTTATTCGGAGTTTCTCATGTGTT	561742	ATTGTCAAATAAAGATTCGGTCAAA	563633	1894	2095	AAGGTACATCTTCCCGCTATG	F
CLB5	AAGACGCGCCCTTGATGGTACAAA	775572	TGCTTTCTTCTCATTTTTCATTTCT	773525	2047	2323	GGTAAAGAGCTAGTTCAACTG	R
BNI1	GATGTGGAAAACTAGAATCCCTTTT	135709	CTACAACTTTTGGATGGAAATCAAC	129153	6560	2282	TTGCTTTGCAGGGCTACTTG	R
BNR1	ACAAGAAATAACCATTCGATTTCAA	41485	CATTGCCCATCTTTATTCAATAGTC	46210	4728	2184	GCCCACACCCATGATTTC	F
SMY1	TATGCTTTTGATGAACGATTAGACA	285985	GGTTTGATGCAACTCTACTATTGCT	288525	2543	2156	TGCCAGTTCCTGAATACTTG	F
KAR9	AAAAGGACGATAATGACGATAATGA	32637	TTTCAGTCATTCATATACACTGCGT	35245	2612	2261	ATCTGACTGCATTTATGCTG	F
BUD6	CTGATATCACAATAGGATTCGGTTC	771976	TATGCAATTTCATTCGTTCTTACAA	768965	3014	2231	TAAAACCAGAAGTTCCAGCCG	F
MRS6	AAGTTGTTGCACATTTGATTTTACA	1031278	TGTAAACAAATCAAATGTACGGATG	1028892	2389		CTTCAAATGACAACTTGCTGC	R

CLN1	TTTTGCTGACAATACATTTTAACGA	662532	CATTATTACTTACGATGGAAAAGCG	664358	1830	1773	TTAGAGGATGGGTCGTCAG	R
CLN2	TCGGTCTCTTCGTTCTTCC	67853	GAGCTTAGGATTTTACCACAACTGA	64655	3198	3144	TTTTCGCCGGTTGAGTGTATC	R
BEM3	ACCACAACTCTAAGAATGTCGAAAG	335884	AATGTTGTCTTGAACCCACTAAAGA	331714	4173	2370	TCCCCGCATCTTTAAAAG	F
BNI4	TTCTTTAGATCACTGCCTGTTCTTT	211563	CCGTACTGTGGGCATATTAGTTTAC	214988	3428	2333	TACTTCGAATGTCCTGGG	F
KCC4	TTTATGTTTTCTGTTATGTTTGCCA	78865	TTTTCAGGTATTTTGGCATGTATTT	81995	3134	2267	TGTGGAGCTATTTTTCAAGTC	F
SWE1	TCACGTGATGTGTATGTTTCTTTTT	79616	GCTTCAAACTTTTTGTCATTTGTCT	76491	3129	2253	AATGCCCTCAGAACCTGAG	F
MIH1	TTTGATACATTGTTCCGGCGTTTAAA	343755	AGACTGCATTAGCAAAAGAGCCATT	341641	2118	1998	TACGGTGCCGCTTACAAATC	F
NAP1	GCAGACATTGACAAGTACATCATTC	526507	TCCGTTATCTTTCCTTTTATAACCC	524769	1741	2071	CAACGAAGCCCTGGATG	F

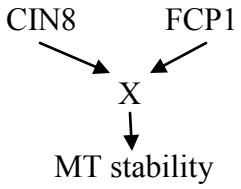
Western blot analysis

10-mL log phase cultures were spun down and resuspended in 1mL ice-cold TE buffer. Total protein lysate was prepared by vortexing cells with glass-beads in 50 µL Yeast Extraction Buffer (0.6% SDS, 10 mM Tris, pH 7.4 with addition of 1 µg/mL leupaptin (Sigma); 2 µg/mL aprotinin (Sigma); 1 µg/mL pepstatin (Sigma); and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). 50 µL Sample Buffer (125 mM Tris-HCL, 20% glycerol, 4.1% SDS, 4% β-mercaptoethanol) was then added, spun, and the supernatant was boiled for 5 minutes. 5 µL was mixed with an equal volume of Laemmli Sample Buffer (Bio-Rad) and loaded on 4-12% Gradient NuPAGE Novex Bis-Tris Mini Gel (Invitrogen), according to manufacturer's instructions. EZ-Run Pre-Stained Protein Marker (Fisher Scientific) was loaded as well. Western blot was performed as described by Solomon et al.[68] using Hoefer TE 22 tank transfer unit and Hybond ECL Nitrocellulose Membrane (Amersham Biosciences). Rat anti-Mrs6 antibody and peroxidase-linked goat anti-rat secondary antibodies were obtained from AbD Serotec. Mouse anti-3-phosphoglycerate kinase (PGK) antibody (Invitrogen) was a gift from P. Lipke and was used as a loading control in order to normalize the results. Peroxidase-linked anti-mouse IgG secondary antibody (GE Healthcare) was used at a 1:5000 dilution. To visualize the results, blots were developed using Amersham's enhanced chemiluminescence (ECL) Detection Reagents (GE Healthcare) in accordance with manufacturer's instructions and detected using Fujifilm LAS-4000. Fluorescence quantification and analysis was done using Fujifilm Multi Gauge software.

Results and Discussion

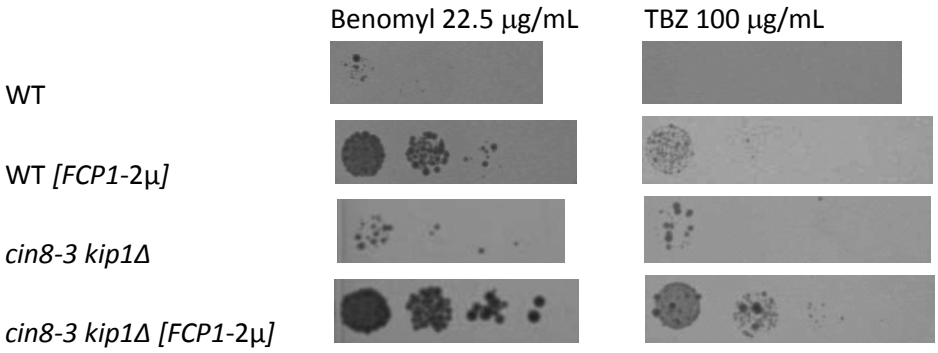
Characterizing the genetic interation between *FCP1* overexpression and CIN8

Before identifying genes needed for *FCP1*'s suppression, we sought to define the genetic interaction between *FCP1* and *CIN8*, the motor required for the suppression to manifest (see Chapter II). To achieve this goal, we grew wild-type and mutant cells overexpressing *FCP1* on plates containing various MT-destabilizing drugs and used the strains' relative sensitivities to draw a putative pathway to the suppression. Because the growth of the double mutant *cin8-3 kip1Δ* overexpressing *FCP1* surpassed the growth level of wild-type cells overexpressing the same gene (see Figure 3.4), we concluded that the following pathway may describe the connection between *FCP*, *CIN8*, and MT stability:



This was the therefore the platform or stage upon which genetic interactions with other genes that came up in our screen were overlaid.

Figure 3.4 Serial dilutions on various MT-destabiliang drugs



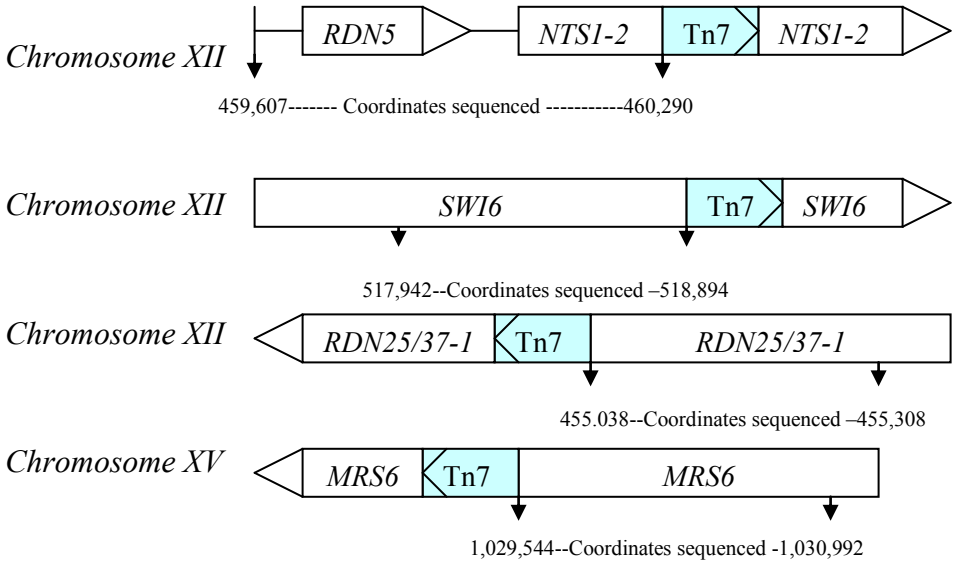
Identification of genes that ameliorate the effect of *FCP1* overexpression on MT stability

Chromosomal regions containing a Tn7 insertion were identified by sequencing of DNA from colonies that gave a positive β-galactosidase activity (not shown) and that lost the ability to grow at the non-permissive temperature in spite of the presence of the multicopy plasmid carrying *FCP1* in those cells. The β-galactosidase test detects colonies carrying in-frame integrations of the *LacZ* fusion into the coding region of the target gene, as the *LacZ* gene of the transposon is promoter-less and requires the target gene's promoter for expression. The *LacZ* gene also imparts a series of stop codons to truncate the gene it is integrated into. By thus creating an effective truncation, the technique allows for the identification of essential genes in haploids that are involved in the process under study, as long as their essential function in the cell was not compromised too much by the truncation.

In addition to the integrations shown in Figure 3.5a, some integrations localized to telomeric sequences that, because of their highly similar sequence, cannot be limited to a specific chromosome (not shown). Furthermore, several colonies carried redundant integrations, reinforcing the relevance of the gene for MT stability.

Figure 3.5 Chromosomal integrations of the Tn7-derived transposon. A. Schematic representation of sites of integration. Figure not drawn to scale. B. Sample sequencing result

A.



B.

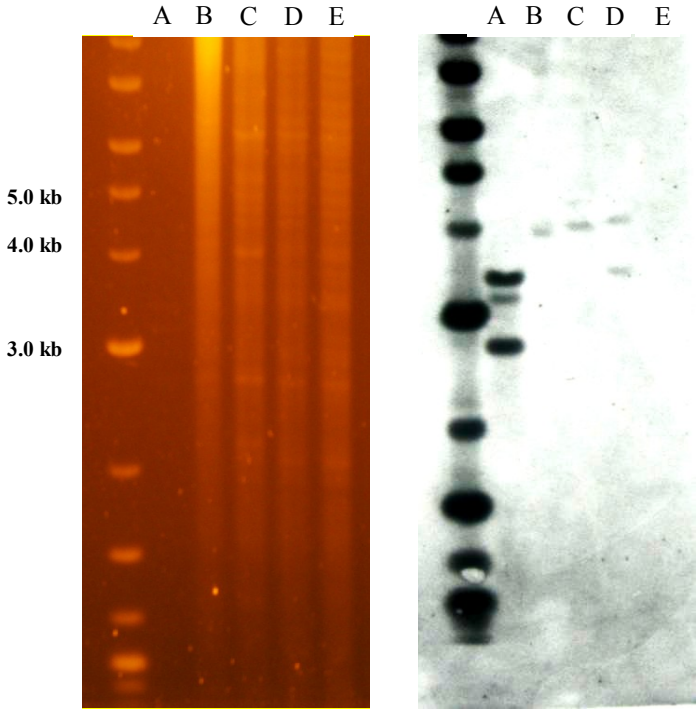
GCCNTAGCAGCTCGAGCGGCCGCCAGTGGTGATGGATATCTGCAGAATTCGGCTTTTCGAGAT
CCCAGCCATTCGAAGCT
ACCAGAAAACAAGACCAAAAGTGAGAATTCTAAGGATAATATCAGCTCAAAGAGGATTAATA
ATTTACAAGATATGAGCC
TGGATTCTGATGCACACAGAGAATTAGGCTCTCCTTAAAAAAACTAAAAATAGATACCTCTGT
AATAGATGCTGAGAGTG
ACTCCACTCCGAATACTGCCAGAGGCAAGCCTAACGATGATATTAATAAGGGCCCTAGCGGC
GACAATGAAAATAATGGC
ACTGATGACAATGACAGAACCGCTGGACCTATCATAACATTCACTCATGACCTAACTTCTGAC
TTTTTAAGCAGTCCACT
GAAAATCATGAAAGCACTACCTTCTCCAGTTGTAAATGATAATGAACAGAAGATGAAACTAG
AGGCATTCTTACAACGGT
TGCTATTTCCAGAAATTCAAGAAATGCCTACATCCCTTAATAAGGACAGCAGTAATAGAAATT
CAGAAGGGGGGAGCTCA
AACCAACAACAACAGCACGTATCATTTGATAGCCTTTTGCAAGAGGTAAACGACGCTTTTCCT
AATACTCAATTAAATCT
TAATATTCCTGTAGATGAGCATTGTGGGCGGACAAAAAAGTCTCAAACTGGACAGAAAAGAT
CCGTCGACGCGGCCATTG
AAGGTAGAAGAGAAAATTTGTACTTCCAAAGCAAGAAGGCCAAGCCGAATTCCAGCACACTG
GCGGCCGTTACTAGTGGA
TCCGAGCTCGGTACCNAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTAT
CCGCTCACAATTCCAC
ACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTACTCA
CATTAATTGCGTTGCGCT
CACTGCCCGCTTTCCAGTCGGGAAACCNGTCGTGCCAGCTGCATTATGAATCGGCCACCCCCC
GGGGAAAGGGNGGNTTG CGTATTGGGCGCTTTTCCGCTTCCTCGCTNACTGACTNN

Blue: pCR2.1 plasmid DNA
Red: SWI6: chr.XII, coordinates 518289-518894
Violet: Tn7 DNA

Southern blot was performed on colonies containing disruptions to the *SWI6* and *MRS6* genes in order to exclude contributions to restoration of the temperature-sensitive phenotype by additional integrations (see Figure 3.6). We did not test colonies containing the insertions into rDNA regions coded by *RDN25/37-1* and *NTS1-2*, as these loci are present in 100-200 tandem copies in the genome[97] and the systematic sequencing of the yeast genome includes only two of the 100-200 rDNA repeats[98].

Southern blot analysis with a probe specific for the *lacZ* region of the transposon revealed that in colonies containing the triple mutants *cin8-3 kip1*Δ *mrs6::Tn7* and *cin8-3 kip1*Δ *swi6::Tn7* the signal observed is due to one unique transposon insertion. The sizes of the bands correspond to HindIII cut sites that are produced when *MRS6* or *SWI6* contain the Tn7 transposon at the positions determined by sequencing (3.9 and 4.4 kb, respectively). We also tested the triple mutant of *cin8-3 kip1*Δ *yer121w::Tn7* that also came up in the screen, but DNA analysis revealed that the colony contains two copies of the transposon. We therefore did not pursue further studies on this mutant.

Figure 3.6 Agarose gel electrophosesis (left) and Southern blot analysis (right) of the same gel. Genomic DNA from designated strains was digested with HindIII. Lane A: library plasmids containing Tn7 insertions. Lane B: *cin8-3 kip1*Δ *mrs6::Tn7* Lane C: *cin8-3 kip1*Δ *swi6::Tn7* Lane D: *cin8-3 kip1*Δ *yer121w::Tn7* Lane E: *cin8-3 kip1*Δ (negative control).



Verification of the results, expansion of the repertoire of genes that ameliorate the effect of *FCP1* overexpression on the stability of microtubules, and construction of putative pathways to suppression

The merit of our screen design stems from the employment of transposons to generate insertions. In haploids, insertional mutagenesis allows the role of nonessential genes implicated in a process under study to be elucidated. The technique may also identify significant insertions into essential genes, as long as these alter the function of the protein but do not yield a null phenotype. It is therefore safe to suggest that since *MRS6* that came up in our screen is an essential gene, the transposon insertion in that locus possibly

created a compromised version of the gene, one that specifically undermined its previously unidentified role in MT stability.

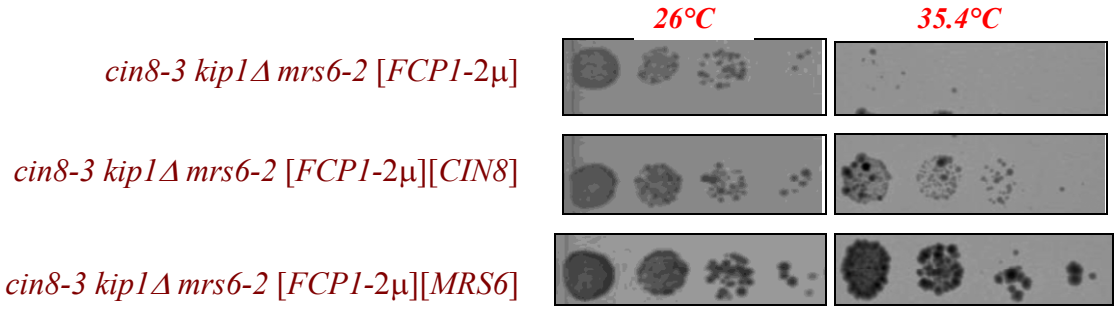
Mrs6p is a rab escort protein: it forms a complex with the small ras-like GTPases Ypt1p and Sec4p and presents them to protein geranylgeranyltransferases type II for prenylation[99]. The association between Mrs6p and the GTPases is required for their prenylation, which in turn allows for membrane attachment and vesicular traffic between the endoplasmic reticulum and the golgi[100].

In higher eucaryotes, rab GTPases can mediate long-range vesicular transport along microtubules by binding directly to motors or by recruiting motor adaptors, and this association was shown to regulate the activity of microtubule-dependent motors[101]. In yeast, blocking of prenylation was shown to lead to defects in the membrane attachment of Ypt1p and Sec4p[102], and shortly after turning off of *YPT1* gene transcription, abnormally elongated cytoplasmic microtubules and asymmetrically stained spindles were observed. After 28 hours, loss of *YPT1* function led to arrested cells that exhibited a complete disorganization of microtubules[103].

Since *MRS6*, the rab GTPases mentioned, and proteins of the rab prenylation machinery are all essential genes, we could not delete these cassettes to further elucidate the role of vesicular transport in microtubule stability. Instead, we obtained a strain containing the temperature-sensitive allele *mrs6-2* that was shown to exhibit reduced prenylation of Ypt proteins, even at permissive temperatures[104]. We amplified the locus containing the mutation by PCR and introduced it to our double mutant in order to confirm the results of the screen. Separately, we also knocked out nonessential genes, such as *VPS21*, *SLT2*, and *BOI2*, that were previously shown to interact genetically or

physically with *MRS6*. Only the triple mutant *cin8-3 kip1Δ mrs6-2* abrogated *FCP1*'s suppression of the temperature sensitivity (see Figure 3.7 and Table 3.2), confirming the results of our secondary screen.

Figure 3.7 Serial dilutions of designated strains at the permissive (left) and non-permissive (right) temperatures



In order to demonstrate complementation, the strains that lost the ability to grow at the non-permissive temperature were transformed with a low-copy plasmid containing a wild-type *CIN8* gene. If wild-type *CIN8* restored growth at that temperature then this suggests that the third mutation does not independently affect the cells when grown at the elevated temperatures but rather, only expresses the phenotype when MT stability is compromised, i.e., in combination with the *cin8-3 kip1*Δ genotype.

Triple mutants that did not abrogate *FCP1*'s suppression, such as those containing *vps21*, *slt2*, or *boi2* deletions, were assayed for the ability of the third mutation to rescue the temperature sensitivity in the absence of *FCP1* overexpression. These triple mutants were streaked on YPD+geneticin plates between 5 to 15 times to ensure the loss of the multicopy plasmid carrying *FCP1* and checked to see whether cells acquire the ability to grow at the non-permissive temperature. This was done to search for genes that *FCP1* inhibits or down-regulates when it is overexpressed or that have a negative effect on MT

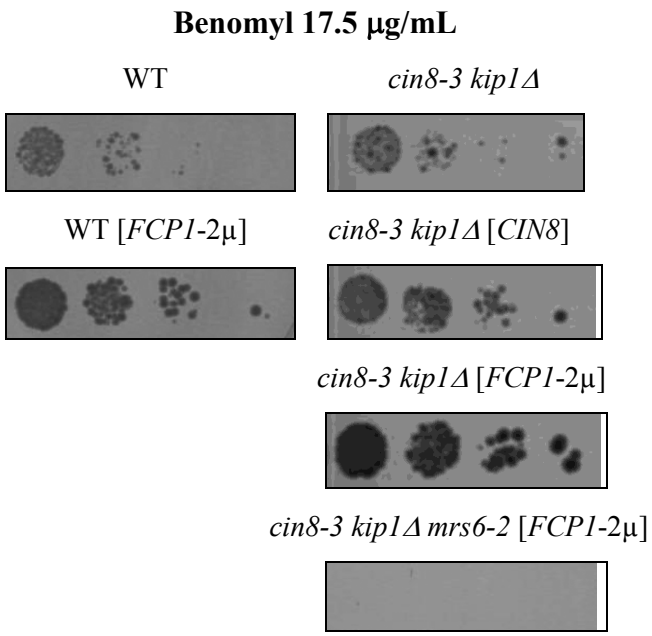
stability in the wild-type. Deletion of genes that restore growth in the absence of *FCP1* overexpression would suggest that in their wild-type condition, these genes have an inhibitory effect on growth. As can be seen in Table 3.3, no such genes were discovered.

Table 3.3 List of triple mutants' growth at conditions assayed.

cin8-3kip1Δ X [FCP1-2μ] X is:	***Growth at non-permissive temp.***	***Rescue by introduction of CIN8***	***Growth in the absence of FCP1-2μ***
mrs6-2	-	+	n/a
vps21Δ	+	n/a	-
slt2Δ	+	n/a	-
boi2Δ	+	n/a	-

Introduction of the *mrs6-2* allele to our double mutant background exacerbated the benomyl sensitivity, suggestive of an additive interaction, placing *MRS6* in a pathway distinct from *CIN8* and *KIP1* that leads to MT stability, and introduction of a wild-type copy of *CIN8* or *MRS6* on a low-copy plasmid rescues these phenotypes (see Figure 3.8). To investigate the relationship between *FCP1* and *MRS6*, we looked at the levels of the Mrs6p protein in double mutants overexpressing *FCP1*. As shown in Figure 3.9, *cin8-3, kip1Δ* cells have approximately a third of the wild-type levels of the Mrs6 protein. *FCP1* overexpression increased these levels in double mutants to about half of wild-type levels.

Figure 3.8 Serial dilutions on YPD- benomyl plates



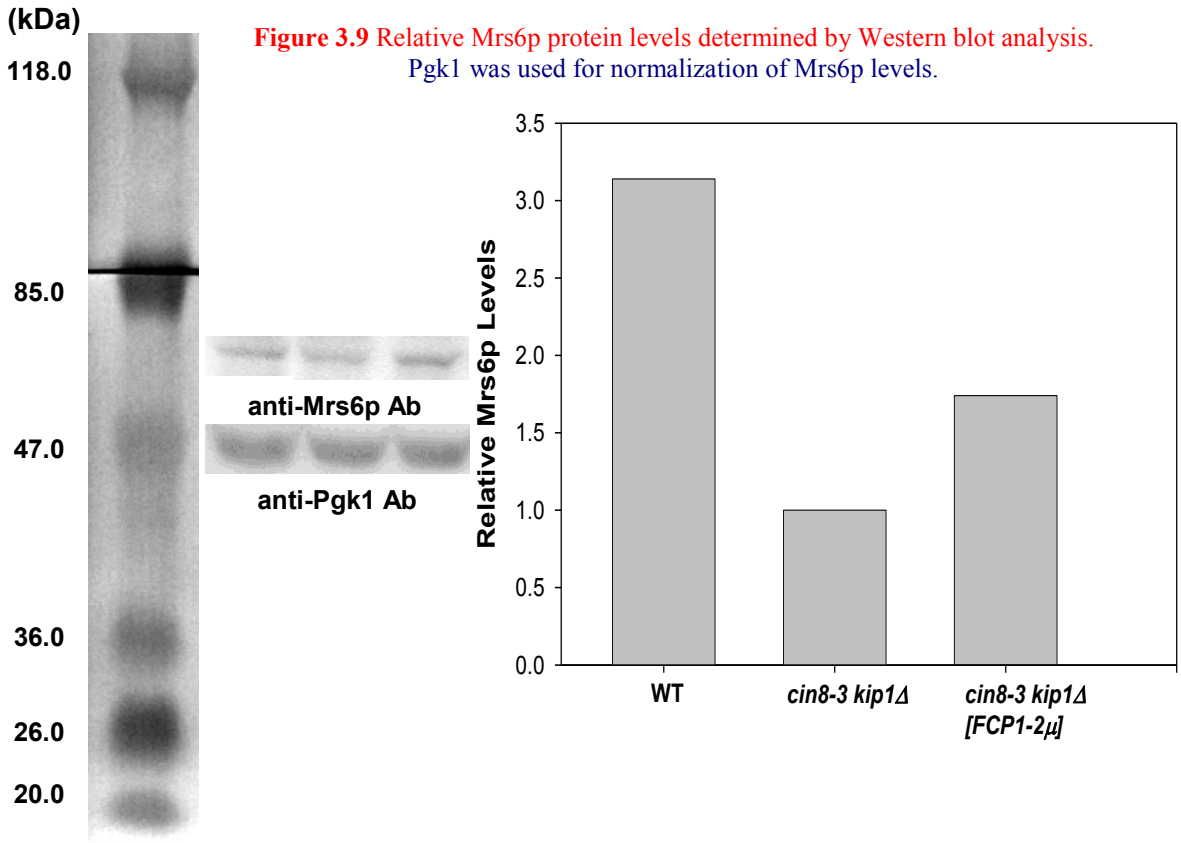
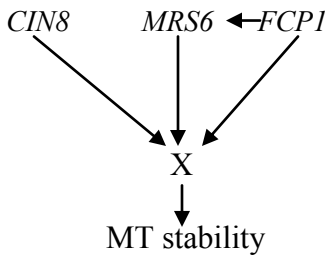


Figure 3.9 Relative Mrs6p protein levels determined by Western blot analysis. Pgk1 was used for normalization of Mrs6p levels.

Since *FCP1* is a general player in transcription, it is reasonable to suggest that its effect on the double mutant is mediated by up-regulating the levels of the Mrs6 protein that was shown to be necessary for the suppression. The following pathway may be drawn to account for these observations, but we cannot exclude other models as some strains that could provide more information could not be constructed.



We therefore decided to focus the rest of our investigations on *SWI6*, the other gene that came up in the screen, and on genes that came up in subsequent tests.

Swi6p is a transcription cofactor which forms heteromeric complexes with the DNA-binding proteins Swi4p and Mbp1p to mediate transcription at the G_1/S transition[105]. More specifically, Swi6p is the regulatory or trans-activator subunit of these complexes. SBF (SCB-binding factor) is the complex containing Swi4p and Swi6p that enhances the transcription of G_1 cyclin genes via SCB elements (**S**wi4/6 **C**ell cycle **B**ox 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 MBC elements (***M****luI* **C**ell cycle **B**ox; ACGCGTNA) and leads to the initiation of a complex transcriptional cascade required for coordinated cell cycle progression[106].

Because of the involvement of Swi6p in both these complexes, we wanted to find out whether one, both, or none of the complexes can be implicated in the suppression. For this, we generated triple mutants of *cin8-3 kip1*Δ *swi4*Δ and *cin8-3 kip1*Δ *mbp1*Δ to see whether these deletions block the effect of *FCP1* overexpression on the temperature sensitivity of our strains.

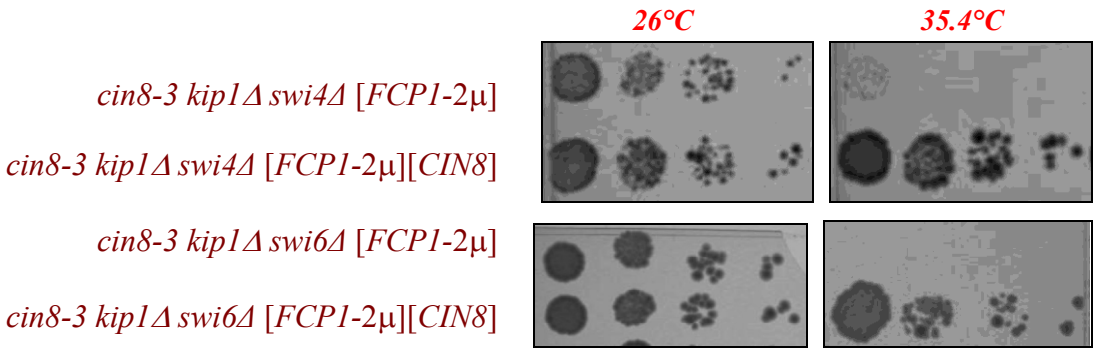
As shown in Table 3.4 and Figure 3.10, *cin8-3 kip1*Δ *swi4*Δ cells, but not *cin8-3 kip1*Δ *mbp1*Δ cells, lost the ability to grow at the non-permissive temperature even in the presence of a multicopy plasmid carrying *FCP1*. Growth at the elevated temperatures was only restored upon introduction of a wild-type copy carrying *CIN8*, suggesting a *swi4* deletion does not independently introduce sensitivity to the strain. These results also

suggest that the SBF complex is a likely candidate through which MT stability is conferred.

Table 3.4 List of triple mutants' growth at conditions assayed.

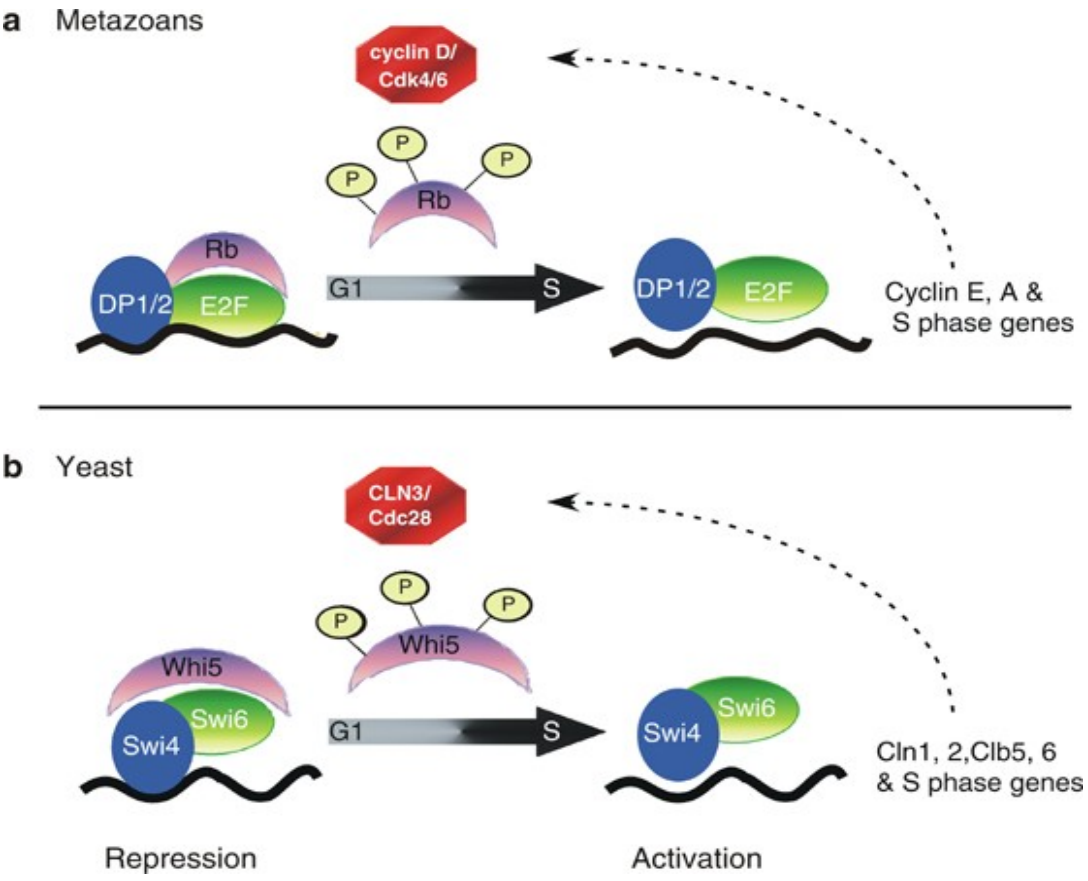
cin8-3kip1Δ X [FCP1-2μ] X is:	*Growth at non-permissive temp.*	*Rescue by introduction of CIN8*	*Growth in the absence of FCP1-2μ*
swi4Δ	-	+	n/a
swi6Δ	-	+	n/a
mbp1Δ	+	n/a	-

Figure 3.10 Serial dilutions of designated strains at the permissive (left) and non-permissive (right) temperatures



The Swi4p/Swi6p (SBF) complex was suggested be functionally analogous to the metazoan DP1/2-E2F complex[107] (see Figure 3.11). This complex is kept in a transcriptionally inactive state by direct interaction with Whi5p (the metazoan Rb equivalent) and this repression is alleviated by hyperphosphorylation of Whi5p by the CDK/cyclin complex Cln3p/Cdc28 at the G_1/S transition[108]. Liberating the SBF complex results in the activation of a transcriptional cascade required for timely S phase entry[109].

Figure 3.11 Comparison of a regulatory pathway controlling the G1/S transition in metazoans and yeast. Adapted from Cooper (2006)



We therefore sought to assess whether any of these G1 regulators of the SBF complex are implicated in *FCP1*'s suppression. As can be seen in Table 3.5, however, their deletion did not inhibit *FCP1* from exerting its effect on the temperature sensitivity of our strain.

Table 3.5 List of triple mutants' growth at conditions assayed.

cin8-3kip1Δ X [FCP1-2μ] X is:	*Growth at non-permissive temp.*	*Rescue by introduction of CIN8*	*Growth in the absence of FCP1-2μ*
whi5Δ	+	n/a	-
cln3Δ	+	n/a	-

These results may suggest that the contribution of Swi4p and Swi6p to the suppression may be independent of their role in the G1/S transition. A thorough investigation of the relevance of Swi4p and Swi6p for the *cin8-3 kip1Δ* background will be discussed in Chapter IV.

To explore the possibility that other regulators are implicated in *FCP1*'s suppression and hence relevant to our background, we similarly screened different cyclins for their involvement. 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 (CDK)[110-113], which pairs up with different cyclins to produce variable effects on the cell cycle. Furthermore, strains containing cyclin or *CDC28* deletions were previously shown to be incapable of assembling a bipolar spindle[54]. In addition, as discussed in Chapter I, the dramatic shift in MT dynamics necessary to form the mitotic spindle was shown to take place concomitantly with a rise in cyclin-depenedent (Cdk) activity[52].

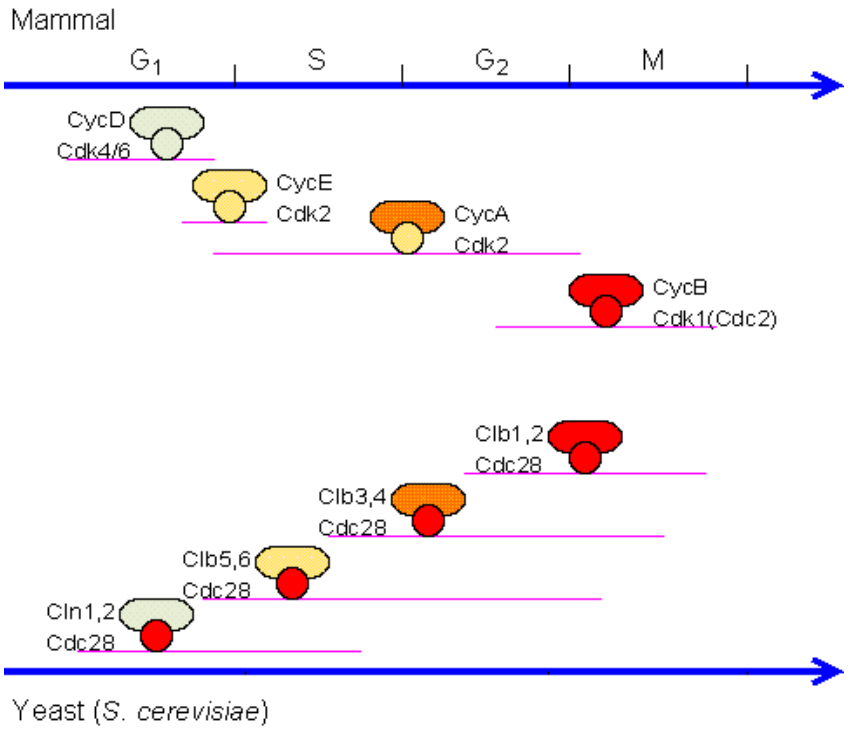
To be active, CDKs have to be associated with a cyclin partner. In budding yeast, the single CDK, product of the *CDC28* gene, is an essential protein that drives the entire cell cycle by successively complexing with a set of nine nonessential cyclins: three Cln

(G_1) cyclins and six Clb cyclins[114]. The cyclins are thought to confer stage-specific functions to Cdc28p: Cln cyclins have functions required for commitment to a new cycle of division, including activation of kinase complexes of Cdc28 and different Clbs through phosphorylation of the CDK inhibitor Sic1[115]. The Clbs are expressed in three transcriptional waves: (1) *CLB5* and *CLB6* are first induced just before the S phase and are implicated in timely activation of DNA replication[116, 117], (2) expression of the mitotic cyclins *CLB3* and *CLB4* peaks upon completion of DNA replication and (3) induction of *CLB1* and *CLB2* peaks around the time of nuclear division[54, 118].

As the periodic expression of these cyclins serves to limit the window of action of Cdc28p to the proper time in the cell cycle[119] (and compartment, as we'll see shortly), we generated triple mutants of *cin8-3 kip1*Δ and different cyclins in order to test for a stage-specific dependency of the suppression (see Figure 3.12).

Figure 3.12 Cyclins involved in cell-cycle specific transitions.

From http://www.web-books.com/MoBio/Free/Ch8A.htm

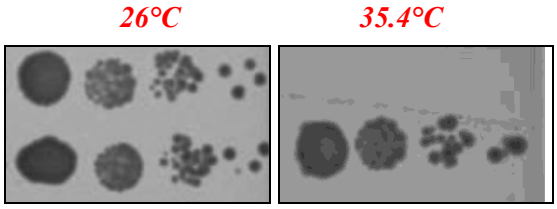


Deletions of different cyclins were introduced individually into *cin8-3 kip1*Δ cells carrying *FCP1* on a multicopy plasmid. As before, we looked for a deletion that abrogates *FCP1*'s suppression of the temperature-sensitivity and for complementation by a plasmid carrying wild-type *CIN8*. *CLB2* is the only gene whose deletion satisfied these parameters (see Table 3.6; Figure 3.13).

Table 3.6 List of triple mutants' growth at conditions assayed.

cin8-3kip1Δ X [FCP1-2μ] X is:	*Growth at non-permissive temp.*	*Rescue by introduction of CIN8*	*Growth in the absence of FCP1-2μ*
cln1Δ	+	n/a	-
cln2Δ	+	n/a	-
clb1Δ	+	n/a	-
clb2Δ	-	+	n/a
clb3Δ	+	n/a	-
clb4Δ	+	n/a	-
clb5Δ	+	n/a	-
clb6Δ	+	n/a	-

Figure 3.13 Serial dilutions of designated strains at the permissive (left) and non-permissive (right) temperatures



In our assays for MT stability we often switched to another benzimidazole derivative other than benomyl, namely, thiabendazole (TBZ), if its effect on the triple mutants was more pronounced. The phenomenon in which a mutation makes a genetic background resistant to a specific drug while remaining sensitive to other compounds within the same family is well established[120]. For example, deletion of genes that have

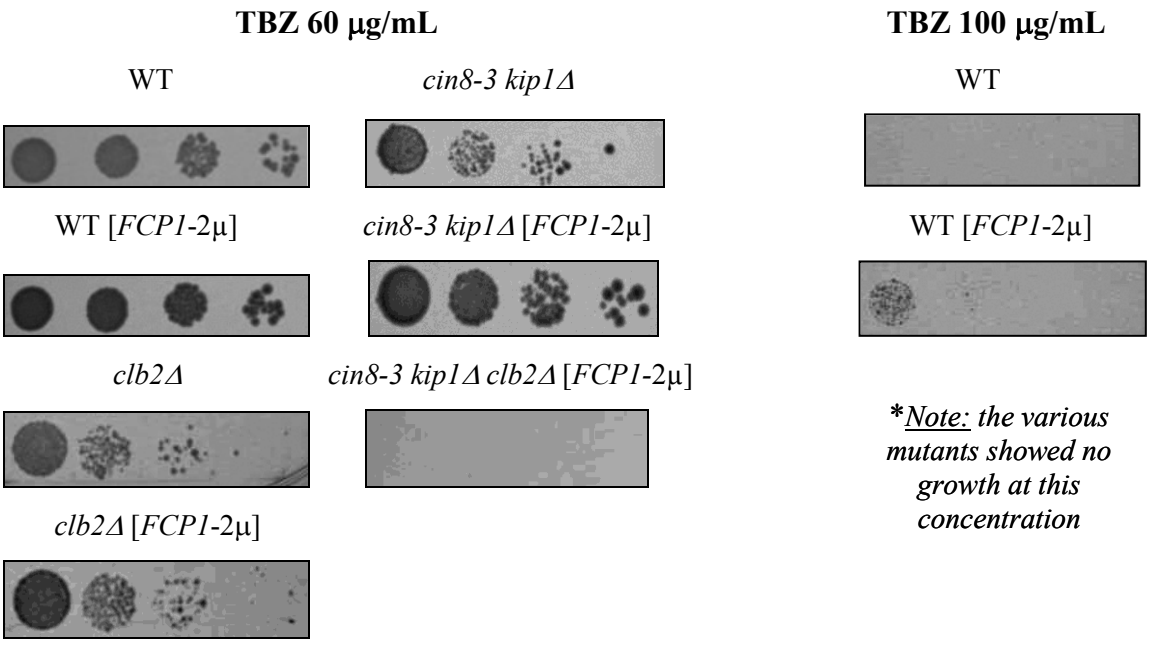
pleiotropic effects often indirectly impair a specific drug's uptake or permeability system, making cells insensitive to the drug. Variations were previously reported in the sensitivity of different mutants to drugs, differences rooted in carrier-mediated systems such as ABC and MFS transporters that underact or overact in the mutants to expel toxic compounds[121]. Since many of the genes that came up in our screen are involved in general or phase-specific transcription processes, it is reasonable to expect that the activity of some transport systems may also be affected.

We therefore used benzimidazole drugs interchangeably in our screens for MT stability, as the general mechanism of MT disruption among members of this family is similar: the high affinity of the drug to the αβ-tubulin heterodimer leads to the dimer's sequestration. This results in a low rate of MT assembly and incorporation of benzimidazole-bound heterodimers into assembling MTs, arresting polymerization and preventing elongation[122].

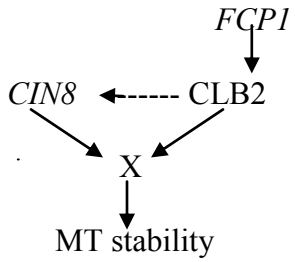
FCP1 overexpression does not alleviate the TBZ sensitivity of *clb2Δ*, suggesting that *FCP1* overexpression may act through Clb2p to stabilize microtubules, as it does not bypass the need for the protein under microtubule-destabilizing conditions (see Figure 3.14). Furthermore, a single *clb2* deletion increases the sensitivity of WT cells to the microtubule-destabilizing drug approximately to the same degree as that seen in the double mutant *cin8-3, kip1Δ*. Although in regards to TBZ sensitivity, the single *clb2* deletion phenocopies *cin8-3 kip1Δ*, their combination in the triple mutant produces enhanced sensitivity to the same concentration of drug, more than the sum of their individual contributions. These results, together with those of Figure 3.13, point to a synergistic interaction operating between *CLB2* and the motors: when *FCP1* is

overexpressed, the motor mutants and the *clb2* deletion separately show only reduced fitness at elevated TBZ concentrations. Their combination in *cin8-3 kip1Δ clb2Δ* triple mutant is deleterious under the same conditions.

Figure 3.14 Serial dilutions on YPD- thiabendazole (TBZ) plates.



According to these results, the following scheme may be drawn:



A yeast two-hybrid interaction was documented for Fcp1 and Cdc28, the kinase binding partner of Clb2[111], providing support for the above model. Furthermore, there is evidence to support the convergence of components of the motor and CLB2 pathways,

indicated by the broken arrow in our model. *clb2Δ* mutants were previously shown to have a diminished capacity to separate the SPBs[54], a phenotype that's in common with *cin8-3 kip1Δ* mutants grown at the non-permissive temperature. More recently, Chee and Haase[110] presented evidence that the Clb2/Cdc28 complex promotes SPB separation and spindle assembly via the kinesin-5 motors Cin8p and Kip1p. They showed that the motors are phosphorylated directly by the Clb2/Cdc28 complex *in vitro* and *in vivo* and that this phosphorylation plays a role in promoting SPB separation and spindle assembly. They also provided evidence that the complex does not regulate Cin8p and Kip1p protein abundance and localization, although in another study it was proposed that Clb2/Cdc28 kinase activity regulates SPB separation indirectly by preventing the motors' degradation. Specifically, the Clb2/Cdc28 complex was shown to phosphorylate Cdh1, an activator of the anaphase-promoting complex (APC). This phosphorylation inhibits the activity of the ubiquitin ligase, preventing the ubiquitination of Kip1p and Cin8p and their subsequent degradation[123]. A deletion of *clb2* alone, however, has no effect on the motors' degradation[124]. These seemingly conflicting studies may imply that the Clb2/Cdc28 complex may be dissected functionally: while Clb2p is the principal factor for regulating the activity of the motors, Cdc28 may have a more dominant role in the motors' stability. We will revisit this concept of functional dissection later in the discussion.

Still, synergism suggests that there may be alternate route/s to microtubule stability, mediated through *CLB2* yet independent of *CIN8* and *KIP1*. To elucidate such pathways, we first investigated some distinguishing characteristics of Clb2p relative to other cyclins. Clb2p is the only cyclin that localizes as a double ring to the mother-bud neck in late anaphase, in addition to its nuclear localization[125]. This targeting to the

mother-bud neck 1) is mediated by Bud3p; 2) requires an intact septin ring; and 3) is independent of the nucleo-cytoplasmic distribution of Clb2p as well as its SPB and mitotic spindle localization[114]. Bud-neck localized Clb2p was shown to have a role in cell polarity and bud morphogenesis[125]. We therefore screened other proteins that localize to the bud neck that have a role in similar processes (some of which interact physically with Clb2p), as well as regulators of the Clb2/Cdc28, for their involvement in the suppression.

Proteins at the bud neck can be classified into septin-interacting proteins and protein regulators of the actomyosin ring. We tested proteins from both classes for their ability to interfere with *FCP1*'s suppression (see Table 3.7 and Table 3.8; Figure 3.15). Septins are protein scaffolds that form a ring-like structure at the mother-bud neck and recruit a plethora of proteins involved in cytokinesis, chitin decomposition, cell polarity, spindle-alignment checkpoint, and bud-site selection. The actomyosin ring is responsible for the physical contraction that separates the two cells. Its role in cytokinesis is presumed to act downstream of the septins[126].

SWE1, which was also tested, encodes a protein kinase that inhibits Cdc28p through phosphorylation of a conserved tyrosine residue, Y19, delaying mitosis. This phosphorylation is reversed by the phosphatase Mih1p, an action that promotes mitosis[127]. Since the immediate substrate of these protein regulators is Cdc28 and not Clb2p, the fact that *SWE1* and *MIH1* were not implicated in *FCP1*'s suppression (see Table 3.8) may suggest that *CLB2* is the main thread through which the suppression is transmitted. The binding partner Cdc28, though is the catalytic subunit, may only be secondary in affecting the suppression. In this case too, we may be observing the functional dissection of the Clb2/Cdc28 complex.

Table 3.7 Selected protein regulators of the actomyosin ring

List of triple mutants' growth at conditions assayed.

cin8-3kip1Δ X [FCP1-2μ] *X is:*	*Growth at non-permissive temp.*	*Rescue by introduction of CIN8*	*Growth in the absence of FCP1-2μ*
bud6Δ	+	n/a	-
bnr1Δ	+	n/a	-
bni1Δ	+	n/a	-
bem3Δ	+	n/a	-

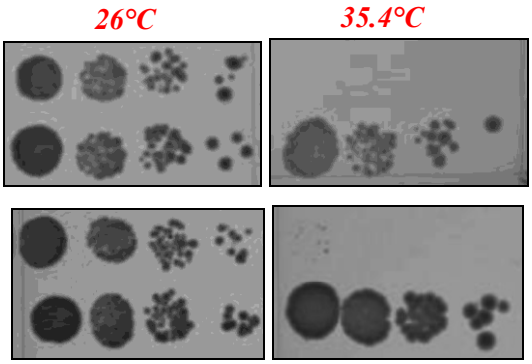
Table 3.8 Selected septin-interacting proteins and their regulators

List of triple mutants' growth at conditions assayed.

cin8-3kip1Δ X [*FCP1*-2μ] *X is:*	*Growth at non-permissive temp.*	*Rescue by introduction of CIN8*	*Growth in the absence of FCP1-2μ*
bud3Δ	+	n/a	-
bni4Δ	+	n/a	-
elm1Δ	-	+	n/a
hsl1Δ	-	+	n/a
gin4Δ	+	n/a	-
kcc4Δ	+	n/a	-
swe1Δ	+	n/a	-
mih1Δ	+	n/a	-

It is interesting that Elm1p and Hs1p, two septin-dependent kinases, came up in our secondary screen (see Figure 3.15), yet none of the proteins we assayed that interact with the actomyosin ring proved to be implicated in *FCP1*'s suppression. These results are in agreement with the finding that the septins, but not the actomyosin ring, are required for the formation of a microtubule-capture site at the bud neck cortex[128].

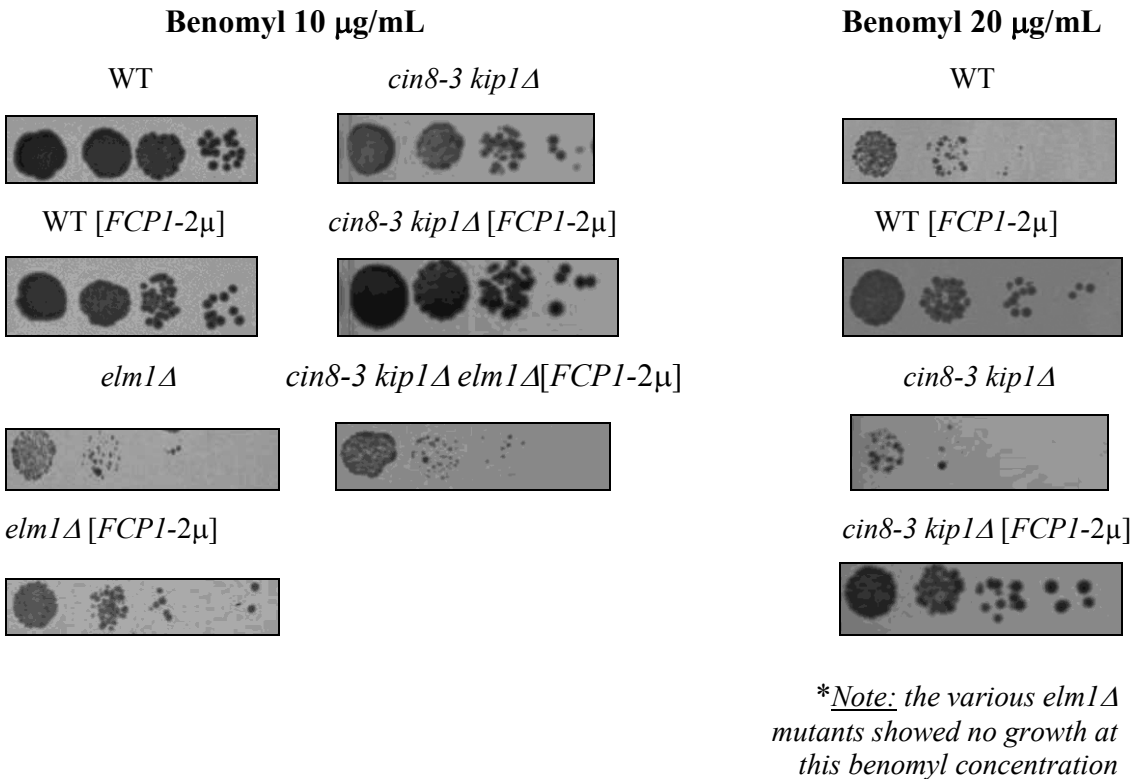
Figure 3.15 Serial dilutions of designated strains at the permissive (left) and non-permissive (right) temperatures



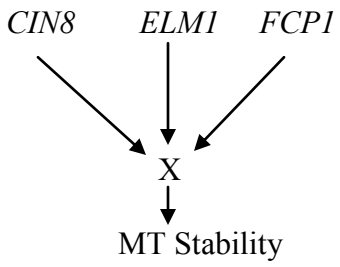
Elm1p and Hsl1p are septin-localized protein kinases which associate with and regulate septin behavior and cytokinesis at the bud neck[129]. They belong to the PAR-1/MARK (microtubule affinity regulatory kinase) kinase family which is conserved from yeast to humans and whose protein members share a similar primary structural organization. Several kinases of this family appear to be at the crossroads of various functions including cell polarity, cell cycle control, microtubule organization and stability at the cortex, or involved in the generation of pulling forces. Additionally, some "Pars" were shown to couple the cell cycle machinery with proper alignment and positioning of the spindle apparatus[130].

A deletion of *elm1* exacerbates the sensitivity of WT cells to benomyl to a far greater degree than that of the double mutant *cin8-3, kip1Δ* (see Figure 3.16). Furthermore, the triple mutant shows the same level of sensitivity to the drug as the single *elm1* mutant, suggesting that in the triple mutant, the *elm1* deletion is the predominant source of cytoskeletal impairment as opposed to the motor mutations, and may therefore act in a pathway distinct from the motors. Additionally, the fact that *FCP1* overexpression alleviates the benomyl sensitivity of the single mutant *elm1Δ* suggests that *FCP1* overexpression may act in a parallel pathway that converges with *ELM1* to stabilize microtubules.

Figure 3.16 Serial dilutions on YPD- benomyl plates.



Based on the growth profiles in Figure 3.15 and 3.16, the following pathway may be envisioned:

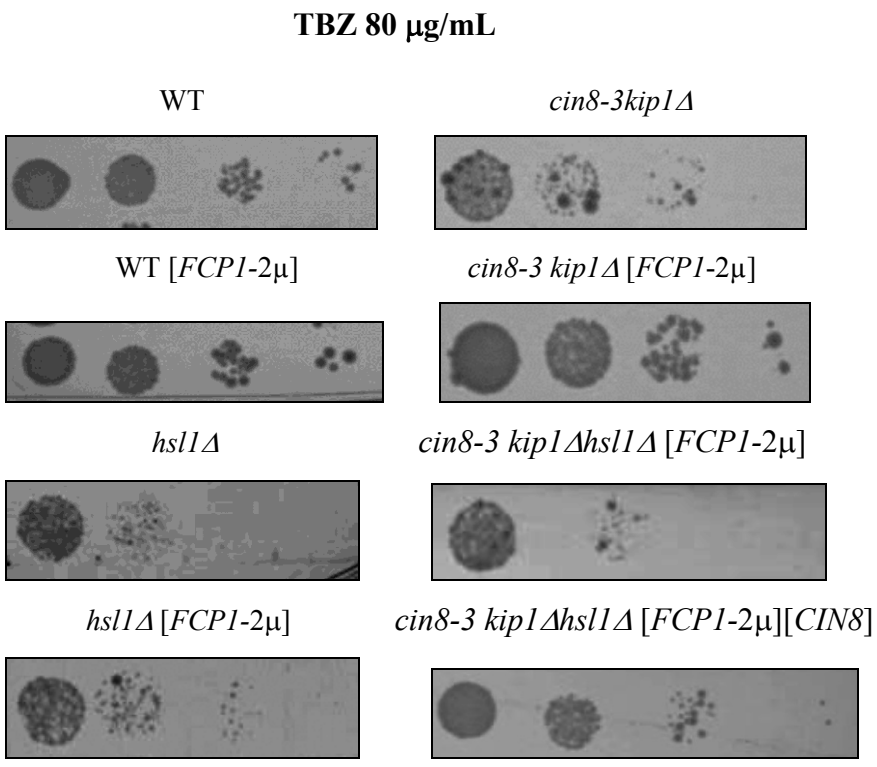


There is evidence to support a genetic and physical interaction between Elm1 and the Clb2/Cdc28 complex. An allele of *ELM1* was isolated in a screen for mutations synthetically lethal with a *cdc28* mutation, affecting the function of Cdc28 at the G_2/M transition[129]. Elm1p was then shown to be required for proper timing of Clb2/Cdc28 kinase activity and peak Elm1 protein levels were shown to correlate with maximal Clb2/Cdc28 activity. Furthermore, the phenotypic consequences of knocking out *ELM1* proved to be much more severe in a Clb2-dependent background[131]. Recently, Elm1p, which similarly localizes as a ring to the bud neck[129], was shown to interact with Clb2/Cdc28 at the bud neck of dividng yeast and to be phosphorylated *in vitro* by the complex[131].

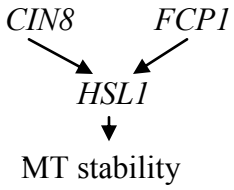
We therefore propose that Clb2p may contribute to MT stability through both its nuclear and cytoplasmic forms: the nuclear form, which was shown to be required for timely anaphase entry[125], may act via *CIN8* and *KIP1*. Cytoplasmic Clb2p, which the authors showed to be required for bud morphogenesis, may contribute to MT stability through the PAR-1/MARK septin-dependent kinases Elm1p and Hsl1p. How these proteins may play a role in MT stability will be discussed in Chapter V.

A deletion of *hsl1* increases the sensitivity of WT cells to TBZ approximately to the same degree as does the double mutant *cin8-3, kip1Δ* (see Fig. 3.17). The triple mutant is not more sensitive to TBZ than either the single *hsl1* mutant or the double mutant *cin8-3, kip1Δ*, suggesting that *HSL1* and the motors may be in the same genetic pathway of microtubule stability (whose order we cannot determine at this point). Furthermore, the fact that *FCP1* overexpression does not alleviate the TBZ sensitivity of the single mutant *hsl1Δ* suggests that *FCP1* overexpression may act through *HSL1* to stabilize microtubules, as it does not bypass the need for the protein under microtubule-destabilizing conditions.

Figure 3.17 Serial dilutions on YPD- thiabendazole (TBZ) plates



According to these results, the following scheme may be drawn:



Genetic data previously suggested that *ELM1* is upstream of *HSL1* [132] and recent biochemical data demonstrated that Elm1 phosphorylates a residue in the activation loop of Hsl1, thereby increasing its activity[133, 134]. However, the effects of the two proteins on cytokinesis were shown to be independent of one another, as data suggests they function in different pathways to control septin assembly and localization[129]. Both proteins, however, were shown to have an effect on cytoplasmic microtubules at the neck. *elm1Δ* mutants were shown to be SPOC (spindle-position checkpoint) deficient[135]. This surveillance mechanism comes into play if cytoplasmic MTs fail to establish correct orientation of the spindle and responds by imposing a mitotic delay by inactivating the mitotic exit network (MEN)[136, 137]. Hsl1 was shown to promote microtubule depolymerization at the bud neck[138]. Moreover, the two proteins were shown to collaborate in a process that is cytoplasmic MT-dependent: Grava *et al.*[139] reported the existence of "two complementary and partially overlapping pathways" to control dynein asymmetry at the spindle poles and associated astral microtubules, pathways that engage Clb2, Elm1, and Hsl1. This asymmetric accumulation of dynein was shown to require cytoplasmic MT contact with the bud neck.

In their paper, the authors present evidence that support the following model: at anaphase onset, when Clb2 reaches maximal levels, dynein is prevented from

accumulating at the mother SPB and its associated MTs by an inhibitory phosphorylation by Cdc28/Clb2. This asymmetry is necessary for spindle orientation, as it ensures that one spindle pole orients toward one end of the cell while the other orients toward the opposite end. Accordingly, dynein is unable to orient the spindle when it localizes to both poles and associated microtubules.

Roughly in parallel, Elm1 and Hsl1 function as spatial cues that recruit dynein preferentially to astral MTs reaching the bud neck cortex. Consequently, dynein pulls one spindle pole strongly toward the bud while the other SPB remains in the mother. During anaphase, the levels of Clb2p drop and dynein is no longer inhibited from accumulating on cytoplasmic MTs of the mother SPB. It consequently becomes symmetric. Symmetrically distributed dynein pulls the two SPBs away from each other and helps the spindle to elongate[140].

Since the specific localization of Clb2p to the bud neck was suggested to contribute to spindle orientation and Cdc28 was shown to interact with the plus end of microtubules[141, 142], we also assayed microtubule binding proteins that were shown to physically interact with Clb2p. In particular, cells lacking Bim1p and Kar9p were shown to position their metaphase spindles randomly[139]. These proteins, however, did not show a genetic interaction with our genetic background (see Table 3.9), but we cannot exclude the possibility that other proteins, possibly essential proteins we could not test by this secondary screen that is based on gene knockouts, are involved in mediating the effects of Clb2, Hsl1, and Elm1 on the spindle. Alternatively, since Hsl1 has attributes characteristic of a microtubule-associated protein, it is not unreasonable to propose that it may directly affect tubulin polymerization and therefore prove to be a bona-fide MAP.

Table 3.9 List of triple mutants' growth at conditions assayed.

cin8-3kip1Δ X [FCP1-2μ] X is:	*Growth at non-permissive temp.*	*Rescue by introduction of CIN8*	*Growth in the absence of FCP1-2μ*
bim1Δ	+	n/a	-
kar9Δ	+	n/a	-
ats1Δ	+	n/a	-
nap1Δ	+	n/a	-

Conclusion

There are certain problems inherent in the employment of DNA microarrays, including the assignment of arbitrary threshold filters to changes in gene expression, the lack of a direct correlation between transcript level and protein levels, and the disregard of post-translational modifications that may activate, inhibit, or sequester the protein. More importantly, microarrays do not address the question of which of the up- or down-regulated genes have an effect on the phenotype. For our investigation we therefore chose insertional mutagenesis, as the advantage of this technique stems from the identification of genes that have a more pronounced role in the suppression of the mutant phenotype, such that pathways enhanced by *FCP1* overexpression or work in parallel to it will be revealed.

The findings in this chapter demonstrate that three genes that came up in our secondary screen cooperate in a process that is implicated in positioning of the mitotic spindle relative to the cleavage apparatus, namely, *CLB2*, *ELM1*, and *HSL1*. The protein

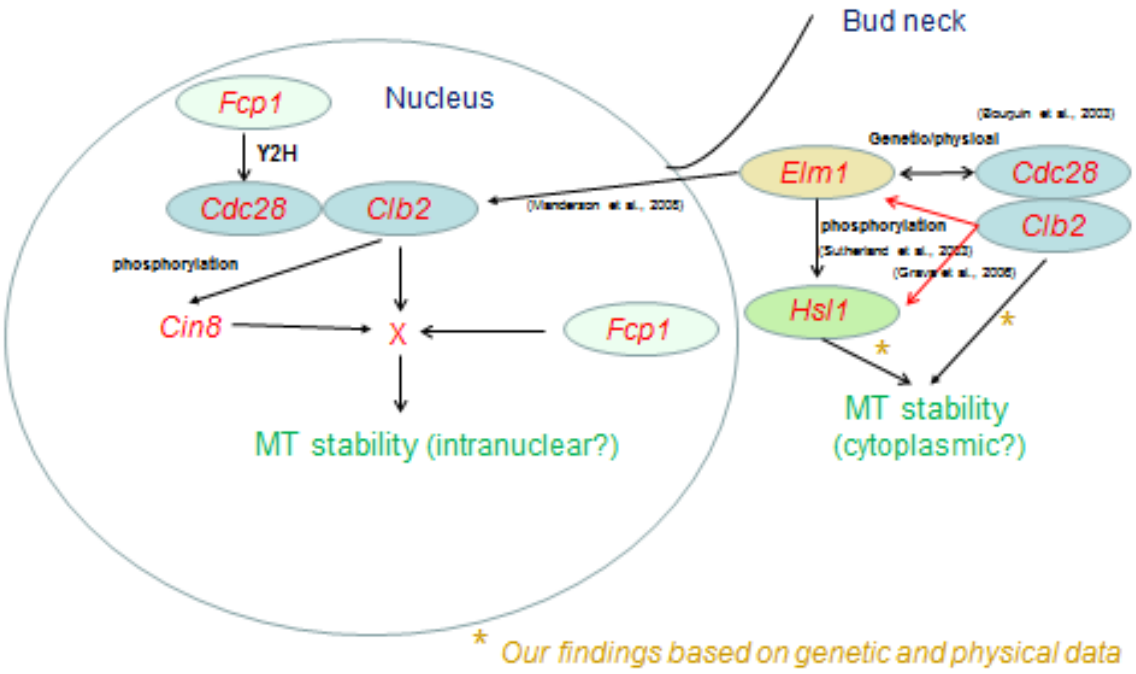
products of these genes serve as spatial landmarks that may regulate MT dynamics at the bud neck, leading to a pulling action on the spindle and a subsequent displacement of the nucleus toward the bud neck. Specifically, the septin-dependent kinase Hsl1 was shown to be required for MT shortening which enables this pulling, and Elm1 may be involved in monitoring or regulating the attachment, based on its implication in SPOC and its capacity to phosphorylate Hsl1p (discussed above).

The induction of microtubule catastrophe and the resultant creation of a pulling force on the spindle were shown to be mediated through Hsl1-dependent phosphorylation events[128]. The single mutant *hsl1* showed enhanced interaction of microtubules at the bud neck for longer periods of time and the MTs were increased in length[128], but it is unknown whether it directly affects tubulin or exerts its function on microtubule-associated proteins. Siller and Doe[138] proposed that since Hsl1 is related to the MARK/Par-1 kinase, which can phosphorylate and inactivate the microtubule-stabilizing Tau protein in neurons, it is possible that Hsl1 may destabilize microtubules by a similar mechanism.

Based on the results and literature presented in this chapter, a model of genetic and physical interactions was constructed (see Figure 3.18). However, we cannot exclude the participation of unidentified components in this pathway. Identification of a putative link between Clb2 and MT stability designated by a question mark in the model will be proposed in Chapter IV. The double arrow between *ELM1* and *CLB2* is indicative of evidence in the literature that suggest that Elm1 can function both upstream of Clb2/Cdc28 and may also be phosphorylated by the complex in a feedback loop[131].

Chapter V will discuss ways in which cytoplasmic events may influence MT stability in the nucleus.

Figure 3.18 A model of genetic and physical interactions. Based on results presented in this chapter and in the literature



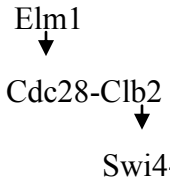
CHAPTER IV

THE INVOLVEMENT OF *SWI6* AND *SWI4* IN MICROTUBULE STABILITY

Introduction

SWI6 and *SWI4* were introduced in the previous chapter as genes whose deletion abrogates *FCP1*'s suppression of the temperature sensitivity associated with *cin8-3, kip1Δ* cells. Swi4 and Swi6 form the binary SBF transcriptional complex which is responsible for G_1-specific gene transcription, whereas Swi6 and Mbp1 (whose deletion did not compromise the suppression) form the MBF complex which mediates S-phase specific transcription (i.e., of genes involved in DNA synthesis and repair). The activator of both these complexes, Cln3, was not shown to be involved in mitigating *FCP1*'s suppression, but the cyclin Clb2 was. Interestingly, the kinase activity of Clb2/Cdc28 was shown to be responsible for specifically displacing SBF (but not MBF) from promoters, leading to the switching off of SBF-dependent transcription at the G_2/M phase [113, 143]. This inactivation was shown to be dependent on physical interaction between Swi4 and Clb2 and subsequent phosphorylation of Swi4 by Cdc28 [113, 144].

Elm1, another gene that came up in our secondary screen, was suggested to be in the same pathway as Clb2 that leads to the inactivation of SBF, as its deletion inhibited the interaction between the Clb2/Cdc28 and Swi4, leading to diminished inactivation of SBF[131]. Furthermore, Elm1p was shown to interact with Cdc28p specifically at the bud neck of dividing yeast. So it seems that Clb2, by virtue of it being the only cyclin that localizes to both the nucleus and the bud neck, has the potential to relay information regarding cell cycle progression from the bud neck to SBF complexes in the nucleus. Based on these documented physical interactions, the following pathway may be envisaged:



In addition to the collective state of Swi4 and Swi6, namely, the SBF complex, establishing individual roles for *SWI4* or *SWI6* in the suppression may also prove to be instrumental in investigating the mechanism of the suppression. This, in turn, requires the exploration of features unique to the proteins. Swi6 localization was shown to be cell-cycle dependent, with nuclear import occurring concomitantly with dephosphorylation of Ser-160 in late M phase throughout G_1, and nuclear export peaking from late G_1 to late M phase and requiring phosphorylation of Ser-160[112]. Activation or repression of Swi6-regulated genes was shown to be independent of Swi6 phosphorylation and hence, localization[145]. Aspartate substitution of Serine 160 significantly impairs nuclear localization, causing Swi6 to remain predominantly cytoplasmic throughout the cell cycle (though Swi6 is not completely excluded from the nucleus), whereas an alanine substitution of the same position leads to constitutive nuclear accumulation of Swi6[145]. These mutations also did not reveal any major changes in cell cycle specific activation or repression of transcription of Swi6-regulated promoters, nor did they have any appreciable effect on the timing or periodicity of SBF- or MBF-driven transcription. From this and other evidence the authors concluded that regulated localization of Swi6 is not required for the activation or repression of Swi6-dependent transcription. Swi4 does not show such differential localization and is found in the nucleus throughout the cell cycle[145].

This chapter will integrate genetic interactions and expression data for *SWI4* and *SWI6* in the *cin8-3, kip1Δ* genetic background and explore the contribution of Swi6 localization for the suppression of the temperature-sensitivity of our mutants.

Materials and Methods

Strains and Media

Strains used in the screen are listed in Table 4.1. Media preparation and yeast genetic techniques were essentially as previously described[63]. Sensitivity to benomyl was tested on YPD-agar to which the desired amount of benomyl (Aldrich Chemical Company, Inc, Milwaukee, WI) was added from 10 mg/ml stock in DMSO. Sensitivity to thiabendazole (TBZ, MP Biomedicals Inc.) was similarly tested and added from 10 mg/mL stock in DMF.

Table 4.1 List of *Saccharomyces cerevisiae* strains and plasmids

Strain	Genotype	Source
MAY589	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101*	M.A.Hoyt
MAY500	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101 TUB2-GFP:URA3*	This study
MAY501	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 ade2-101 TUB2-GFP:URA3 SWI6-mCherry:KanR*	This study
DEY2169	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112*	This study

	ura3-52 ade2-101 trp1Δ	
DEY2000	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ TUB2-GFP:URA3*	This study
DEY2001	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ TUB2-GFP:URA3 SWI6-mCherry:hgh*	This study
DEY3001	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ swi6::KanMX4*	This study
DEY3002	*MAT***a** c*in8-3 kip1::HIS3 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1Δ swi4::KanMX4*	This study

Plasmids

YEp24	2μ *URA3*	NEB**
YEplac112	2μ *TRP1*	NEB**
pMA1208	*CIN8 LEU2 CEN*	M.A. Hoyt
YEplac112-*FCP1*	*FCP1* 2μ *TRP1*	This study
BD1265	*SWI6 LEU2 CEN*	L. Breeden
BD1436	*swi6-S160D LEU2 CEN*	L. Breeden
BD1651	*swi6-S160A LEU2 CEN*	L. Breeden
pAFS125	*GFP(T65)-TUB1:URA3*	A. Straight
pBS34	mCherry:KanR	YRC
pBS35	mCherry:hgh	YRC

*Yeast Genetic Stock Center, University of California, Berkely

**New England Biolabs Inc., Beverly, MA, USA

***See text for details

Strain Construction

Integrative plasmid pASF125 containing the *TUB2-GFP* fusion was linearized by digestion with StuI and transformed into MAY589 and DEY2169 strains according to Davis Lab protocols http://depts.washington.edu/yeastrc/pages/fm_home3.html. The mCherry-KAN or mCherry-HGH cassettes were amplified from their corresponding plasmid using Finnzymes Phusion Hot Start High Fidelity DNA Polymerase (NEB) according to manufacturer's instructions. The primers used to fuse mCherry to the C-terminal of genomic *SWI6* were designed according to instructions in the YRC website above. They are: 5'-CACTGACGAAATGCAAGATTTTTTAAAAAAGCATGC-3' and 5'-AATAAAGTCATAAAAGTTAATGCAATGAAATCACAT-3'. PCR products containing the amplified cassettes were verified through agarose gel electrophoresis and transformed (as before) into wild-type and haploid *cin8-3 kip1*Δ double mutants. Geneticin or hygromycin B-resistant colonies were re-streaked on the same plates. DNA was subsequently purified from triple mutants and confirmation primers (a few hundred base pairs upstream and downstream of *SWI6*, see Table 3.2) were used to confirm the mCherry cassette was integrated into the correct locus.

Fluorescence Microscopy

Fluorescence microscopy was done on Nikon Eclipse 90i using an NA 1.45 Plan Apo TIRF 60X oil-immersion objective. Images were taken with the appropriate filters with an Olympus DP71 camera and processed with the Olympus DP Controller software.

Western blot analysis

10-mL log phase cultures were spun down and resuspended in 1mL ice-cold TE buffer. Total protein lysate was prepared by vortexing cells with glass-beads in 50 µL Yeast Extraction Buffer (0.6% SDS, 10 mM Tris, pH 7.4 with addition of 1 µg/mL leupaptin (Sigma); 2 µg/mL aprotinin (Sigma); 1 µg/mL pepstatin (Sigma); and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). 50-µL Sample Buffer (125 mM Tris-HCL, 20% glycerol, 4.1% SDS, 4% β-mercaptoethanol) was then added, spun, and the supernatant was boiled for 5 minutes. 5-µL was mixed with an equal volume of Laemmli Sample Buffer (Bio-Rad) and loaded on 4-12% Gradient NuPAGE Novex Bis-Tris Mini Gel (Invitrogen), according to manufacturer's instructions. EZ-Run Pre-Stained Protein Marker (Fisher Scientific) was loaded as well. Western blot was performed as described previously[68] using Hoefer TE 22 tank transfer unit and Hybond ECL Nitrocellulose Membrane (Amersham Biosciences). Rabbit anti-Swi6 antibody (gift from the Breeden lab) and peroxidase-linked goat anti-rabbit secondary antibodies were both used in a 1:5000 dilution. Rabbit anti-Swi4 antibody (gift from the Andrews lab) and peroxidase-linked goat anti-rabbit secondary antibodies were used at a 1:5000 and 1:10,000 dilution, respectively. Mouse anti-3-phosphoglycerate kinase (PGK) antibody (Invitrogen) was a gift from P. Lipke and was used as a control in order to normalize the results. Peroxidase-linked anti-mouse IgG secondary antibody (GE Healthcare) was used at a 1:5000 dilution. To visualize the results, blots were developed using Amersham's enhanced chemiluminescence (ECL) Detection Reagents (GE Healthcare) in accordance with manufacturer's instructions and detected using Fujifilm LAS-4000. Fluorescence quantification and analysis was done using Fujifilm Multi Gauge software.

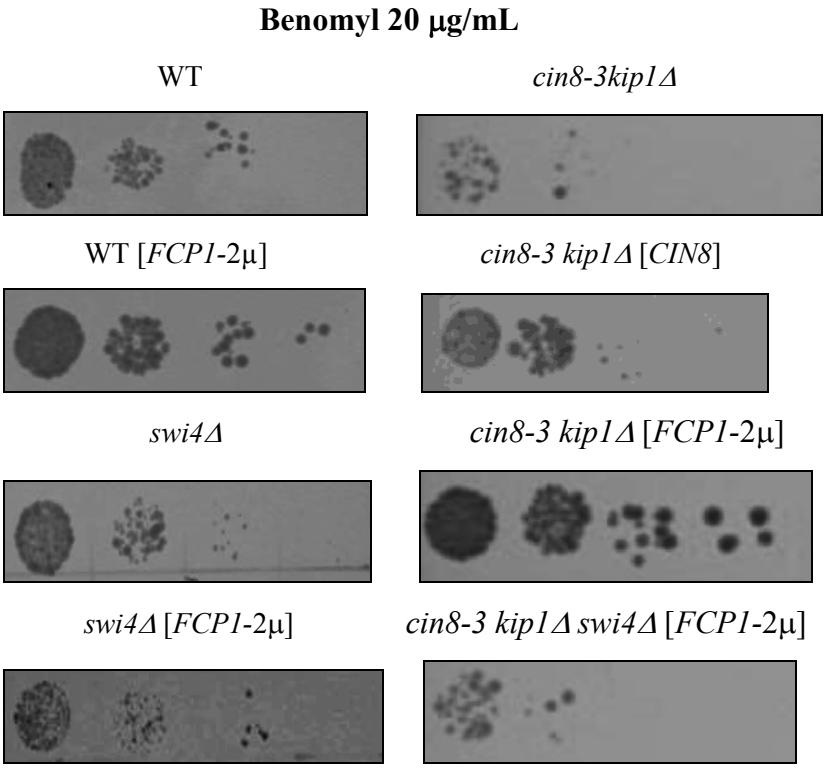
Results and Discussion

Genetic interactions between *SWI4*, *SWI6*, and *FCP1*

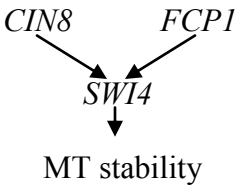
In Chapter III we established that individual deletions of *swi4* and *swi6* in a *cin8-3, kip1Δ* background ameliorates *FCP1*'s suppression of the temperature sensitivity associated with the strain. We demonstrated a genetic interaction between *SWI4* and *SWI6* and our mutants by showing that introduction of a wild-type copy of *CIN8* restores *FCP1*'s suppression. To further our understanding of the genetic pathways that may underlie or give rise to these phenotypes, we examined the growth profiles of strains containing various mutants of *swi4* and *swi6* under microtubule-destabilizing conditions.

A deletion of *swi4* increases the sensitivity of WT cells to benomyl (see Figure 4.1) and *FCP1* overexpression does not restore its growth. This suggests that *FCP1* overexpression may act through *SWI4* to stabilize microtubules, as it does not bypass the need for *SWI4* under microtubule-destabilizing conditions. Furthermore, the triple mutant is not more sensitive to benomyl than the double mutant *cin8-3 kip1Δ*, suggesting that *SWI4* and the motors may be in the same genetic pathway leading to microtubule stability.

Figure 4.1 serial dilutions on YPD- benomyl plates.



According to these results, the following scheme may be drawn:



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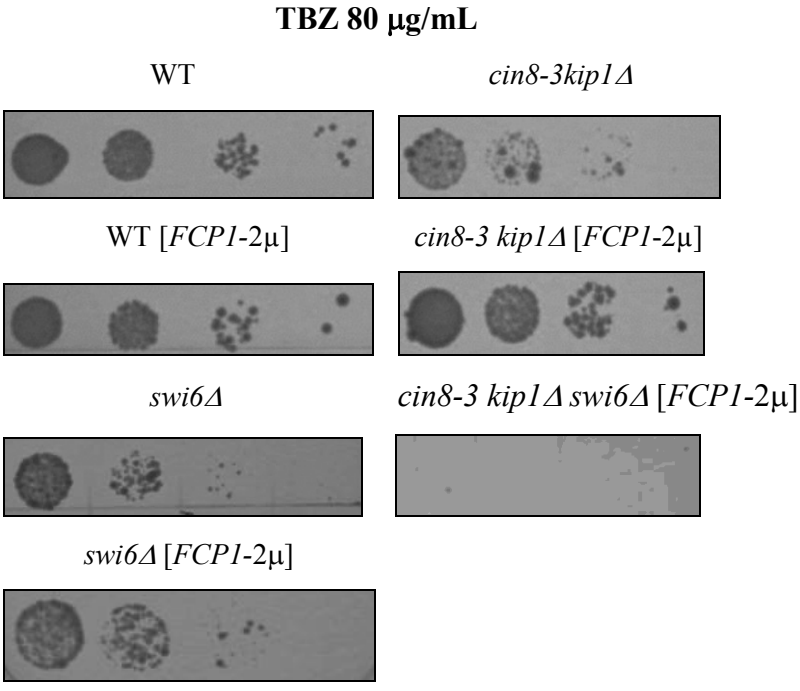
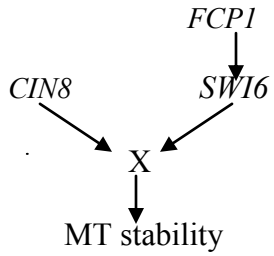
We did the same for *SWI6*. *FCP1* overexpression does not alleviate the TBZ sensitivity of the single mutant *swi6Δ*, suggesting that *FCP1* overexpression may act through Swi6p to stabilize microtubules, as it does not bypass the need for the protein under microtubule-destabilizing conditions (see Figure 4.2). Furthermore, the *swi6* deletion increases the sensitivity of WT cells to the microtubule-destabilizing drug TBZ

approximately to the same degree as that seen in the double mutant *cin8-3, kip1Δ*. Although in regards to TBZ sensitivity, the single *swi6* deletion phenocopies *cin8-3 kip1Δ*, their combination in the triple mutant produces enhanced sensitivity to the same concentration of drug, more than the sum of their individual contributions. These results, together with those of Figure 3.9, point to a synergistic interaction operating between *SWI6* and the motors: when *FCP1* is overexpressed, the motor mutants and the *swi6* deletion separately show only reduced fitness at elevated TBZ concentrations. Their combination in *cin8-3 kip1Δ swi6Δ* triple mutant is deleterious under the same conditions. In fact, no growth of the triple mutants is observed at an even lower concentration, 60 μg/mL thiabendazole, when the single *swi6Δ* mutant and the *cin8-3 kip1Δ* double mutant exhibit growth profiles that almost match those of the wild-type (not shown).

Figure 4.2 Serial dilutions on YPD-thiabendazole (TBZ) plates

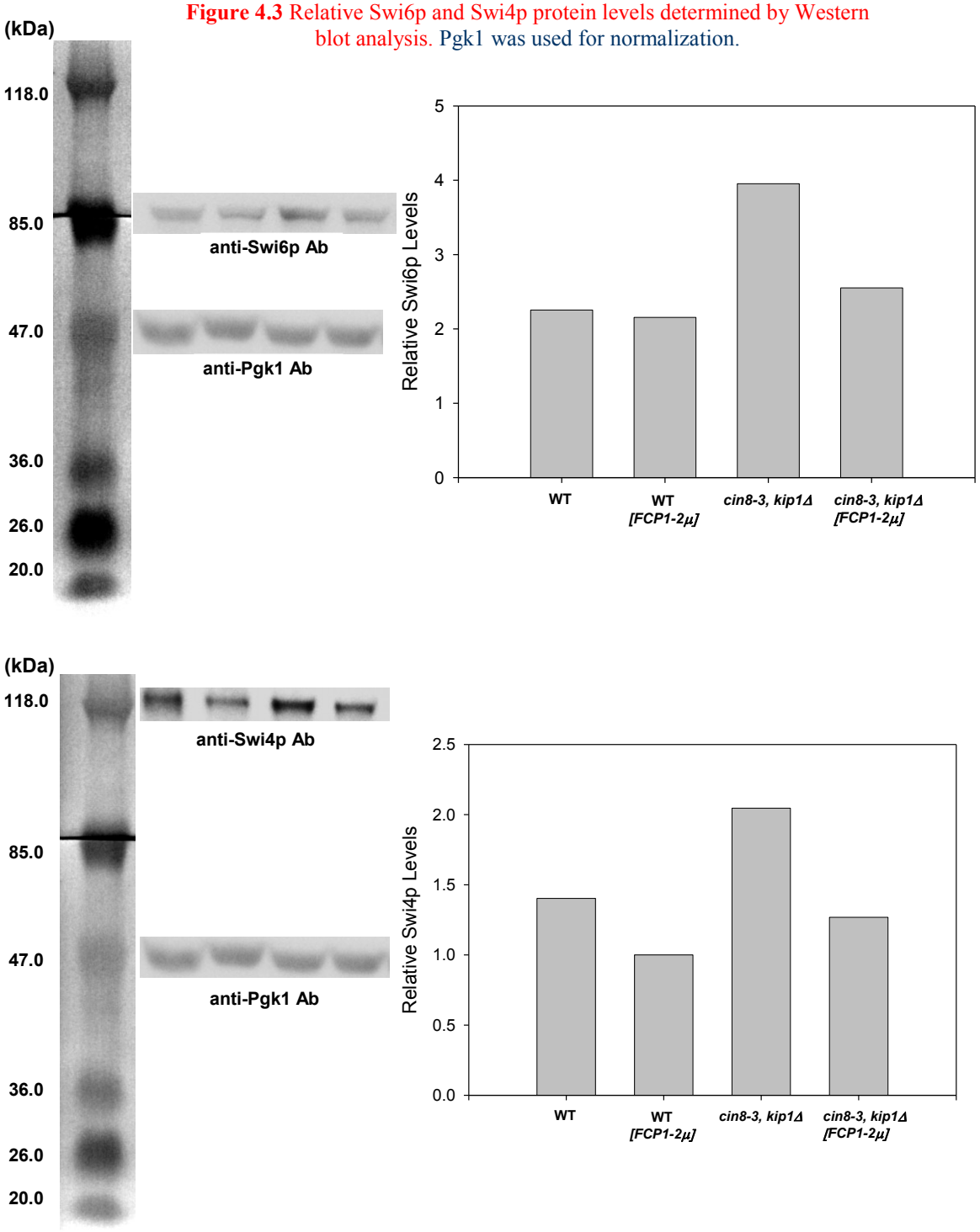


According to these results, the following may be drawn:



We also looked at the levels of the Swi4 and Swi6 proteins in cells overexpressing *FCP1*. As shown in Figure 4.3, *FCP1* overexpression in wild-type cells does not significantly alter the levels of Swi4p and Swi6p as judged by Western blot analysis. The double mutant, however, seems to have elevated levels of the proteins compared to the wild-type. When *FCP1* is overexpressed in the double mutants, the levels of the two proteins seem to come back to roughly normal levels. Furthermore, though Swi4p and Swi6p are present in different amounts in cells, overexpression of *FCP1* in the *cin8-3 kip1Δ* background downregulates Swi4p and Swi6p by the same factor relative to double mutants not expressing it. A common down-regulation factor implies that even though the two proteins are found in different concentrations in cells, only if the two proteins function in a complex will their molar ratios be affected similarly. Our results therefore suggest that *FCP1* overexpression reduces the levels of the SBF complex specifically, as its components Swi4 and Swi6 are downregulated by the same factor in *cin8-3 kip1Δ* cells. The results also propose that the genetic interaction outlined in our model between *FCP1* and *SWI4* or *SWI6* based on the growth profiles on MT-destabilizing drugs (see Figures 4.1 and 4.2) may not necessarily be mediated by the individual genes but by the complex they form. Taken together, the data favors a correlation between SBF complex levels and MT stability.

Figure 4.3 Relative Swi6p and Swi4p protein levels determined by Western blot analysis. Pgk1 was used for normalization.

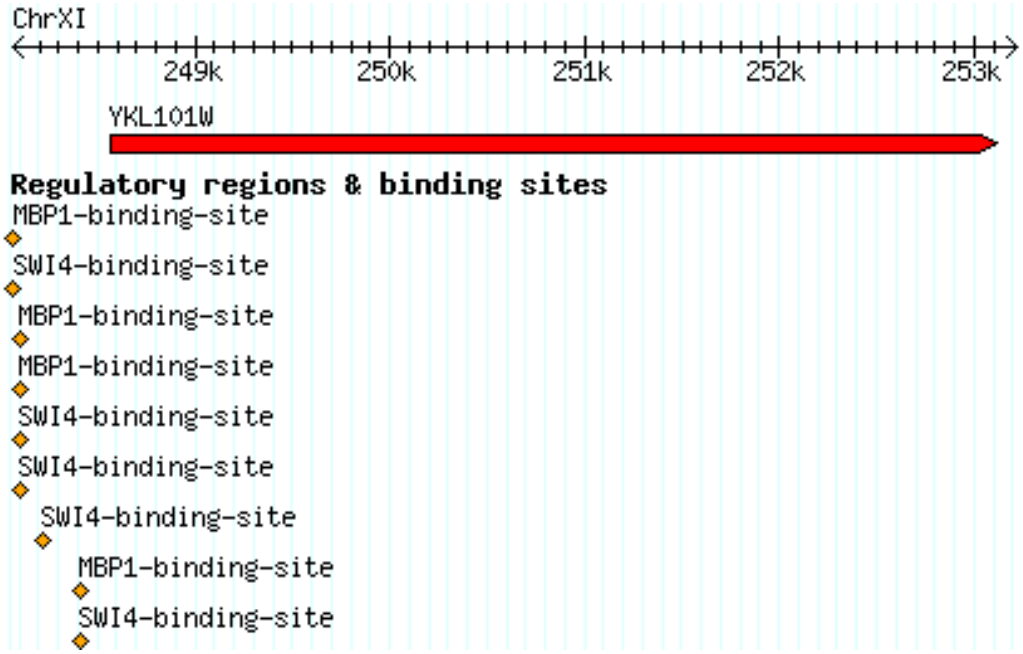


Our immunoblot results present the possibility that the SBF complex may be involved in the suppression. The SBF complex was previously shown to be inhibited at

the G2/M phase by the Clb2/Cdc28 complex[113, 143], a time in which *cin8-3 kip1Δ* cells arrest at the non-permissive temperature when shifted before spindle formation (reviewed in Chapter III). Since a yeast-two hybrid interaction was reported for Fcp1 and Cdc28[111], it is reasonable to propose that SBF restriction at the G2/M phase, mediated by the Clb2/Cdc28 complex, may be involved in the *FCP1*'s suppression.

Still, complete inhibition (i.e, deletion) of individual components of the SPB complex (either *swi4Δ* or *swi6Δ*) was shown to be counterproductive to the suppression. Our results may therefore suggest that a limited window of SBF activity may be required for the suppression of the temperature-sensitivity of our mutants to manifest. Reduced Swi6p levels due to *FCP1* overexpression would result in attenuation of SBF activity, leading to its preferential association with specific promoters, like promoters whose combinatorial activity may produce the effect of MT stability. In other words, a specific activity of SBF brought about by *FCP1* overexpression may re-route the transcriptional program, preferentially transcribing genes necessary for the suppression, genes bearing Swi4- (and hence, SBF) binding sites in their promoter, such as *HSL1* (see Figure 4.4), which was shown to promote microtubule dynamics at the bud neck[138]. Another gene that contains such sites in is *NUD1*, a protein that was shown to have roles in anchorage of MTs and regulation of the mitotic exit network[146]. However, since *NUD1* is an essential gene, we could not delete it from our background in order to test for its involvement in the suppression the way we did thus far.

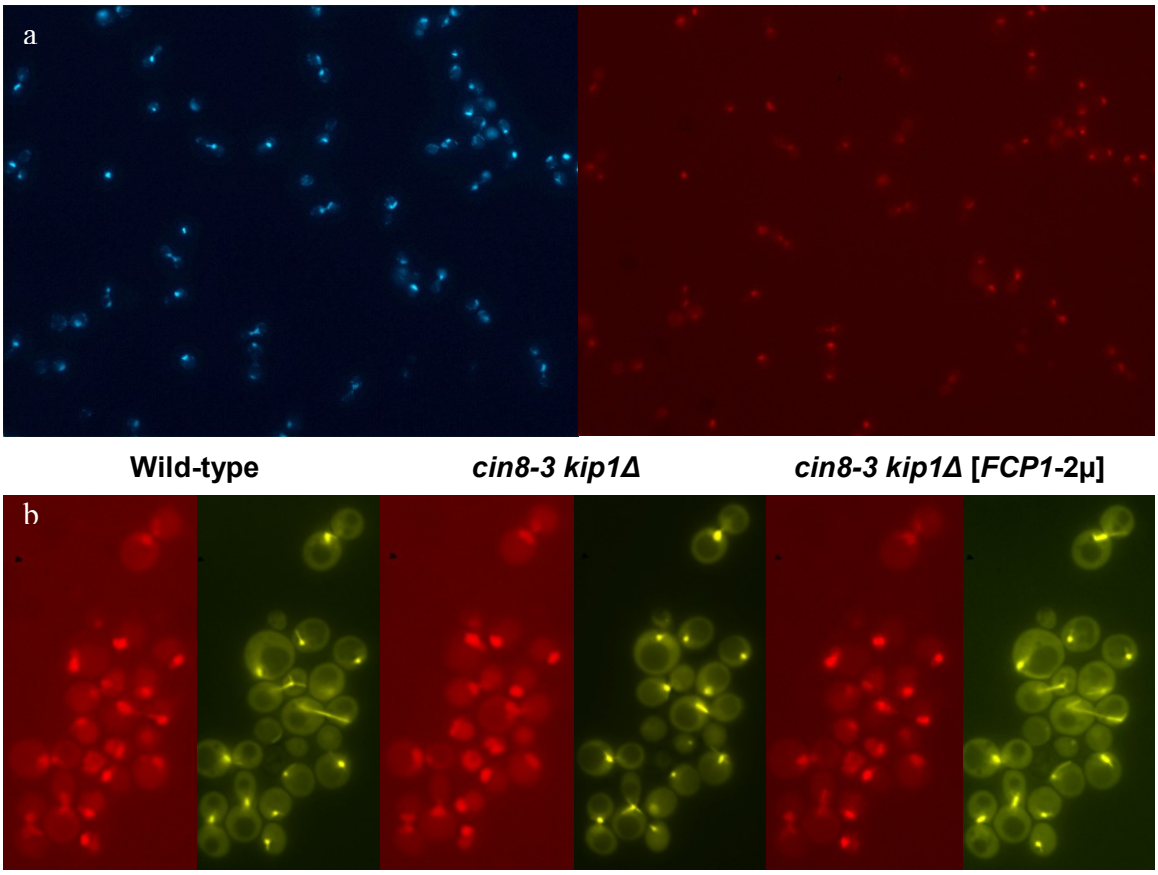
Figure 4.4 Numerous Swi4p binding sites in the promoter region of the *HSL1* (YKL101w) gene. Swi6p is not represented as it is the regulatory component of the SBF complex and Swi4 physically binds to promoter DNA. Taken from *Saccharomyces* Genome Database



***FCP1* overexpression does not alter the cellular localization of Swi6-mCherry**

Because of the cell-cycle specific nucleo-cytoplasmic shuttling of Swi6, we wanted to explore the possibility that *FCP1* overxpression alleviates the temperature-sensitivity of *cin8-3 kip1Δ* cells by altering the localization of the protein. We tested for this in two ways. First, we compared the localization of Swi6-mCherry in wild-type, *cin8-3 kip1Δ*, and *cin8-3, kip1Δ* cells overexpressing *FCP1* (Figure 4.5). In asynchronous populations, Swi6 appears to be predominantly nuclear as seen using DAPI staining. This is expected as the G_2/M phase during which Swi6p is cytoplasmic is transient and so a small fraction of cells will be found in it at any given time.

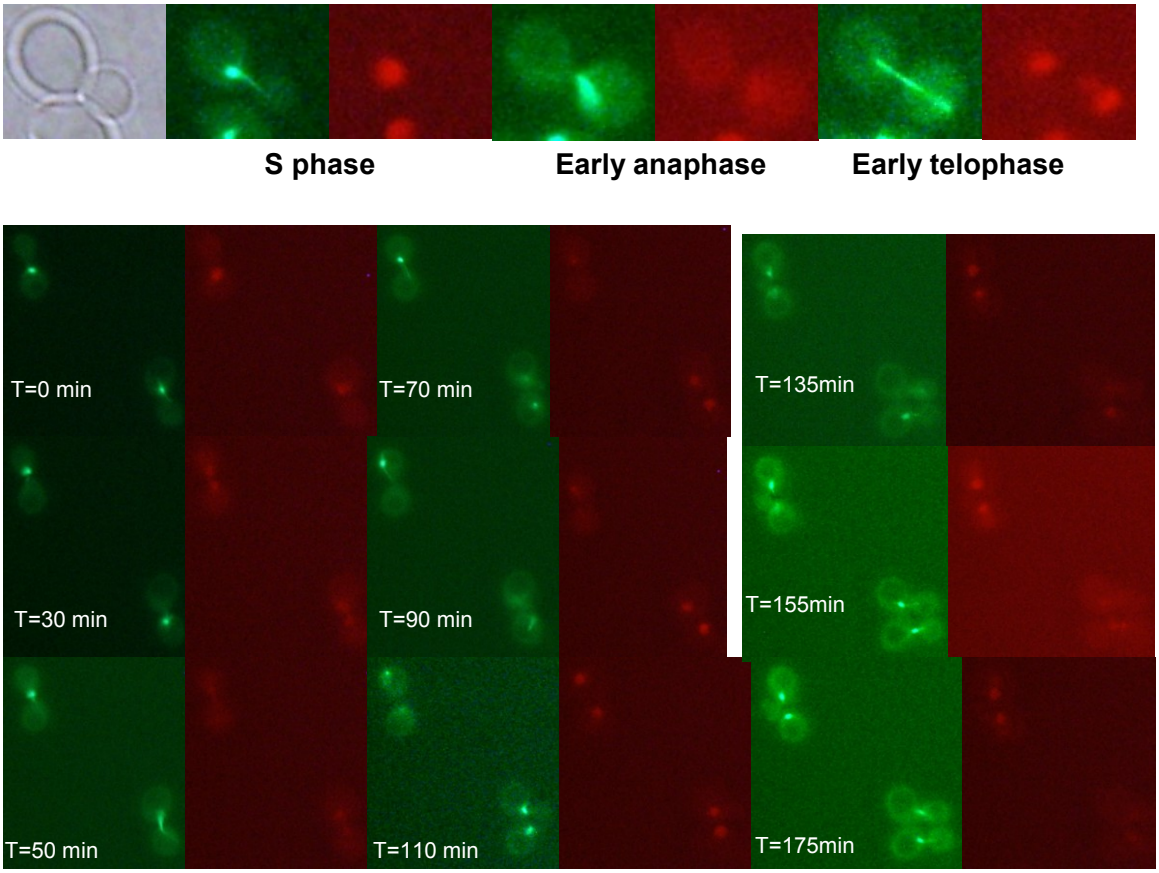
Figure 4.5 Swi6p-mCherry localization in an asynchronous poputation. a) Swi6-mCherry localization corresponds to that of DAPI staining, confirming its nuclear localization throughout most of the cell cycle. b) Swi6p-mCherry localization relative to GFP-Tub2p in wild-type, *cin8-3 kip1Δ* cells, and *cin8-3 kip1Δ* cells overexpressing *FCP1*



To observe the protein's cytoplasmic localization we therefore had to do a time-course analysis of Swi6-mCherry localization. Although we were able to observe cytoplasmic localization from early anaphase (that is characterized by a short, 2μ spindle until early telophase (the point in which the spindle has reached its maximum length), we were unable to observe aberrant localization or changes in the timing of entry or exit of Swi6p from the nucleus in the different strains we examined (wild-type, *cin8-3 kip1Δ* cells, and *cin8-3 kip1Δ* cells overexpressing *FCP1*, see Figure 4.6*)*. All three strains

assayed showed localizations similar to those predicted in the literature with nuclear import occurring during late M phase throughout G_2, and nuclear export peaking from late G_2 to late M phase[147].

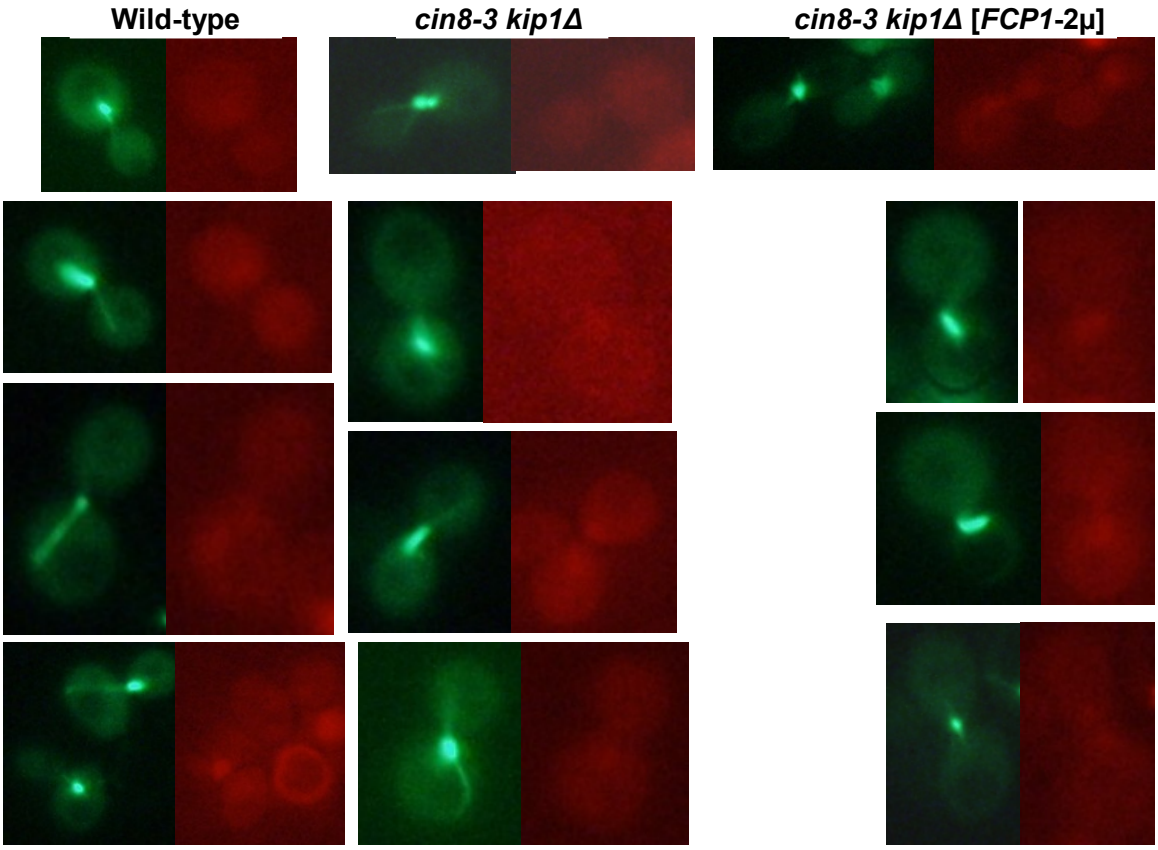
Figure 4.6 Time-course analysis of Swi6p-mCherry localization relative to GFP-Tub2p in *cin8-3 kip1Δ* cells overexpressing *FCP1*. Representative images. Swi6p-mCherry localization in wild-type and *cin8-3 kip1Δ* cells (not shown) was similar.



To further confirm that Swi6-mCherry localization does not change significantly between the strains, we took representative images of the cell-cycle phases (that the GFP-Tub2 protein was a marker for) in which Swi6-mCherry is cytoplasmic (see Figure 4.7).

Again, we did not observe significant differences in the cell-cycle stages that showed cytoplasmic localization.

Figure 4.7 Cytoplasmic localization of Swi6p-mCherry from G2/M to late anaphase. Selected images



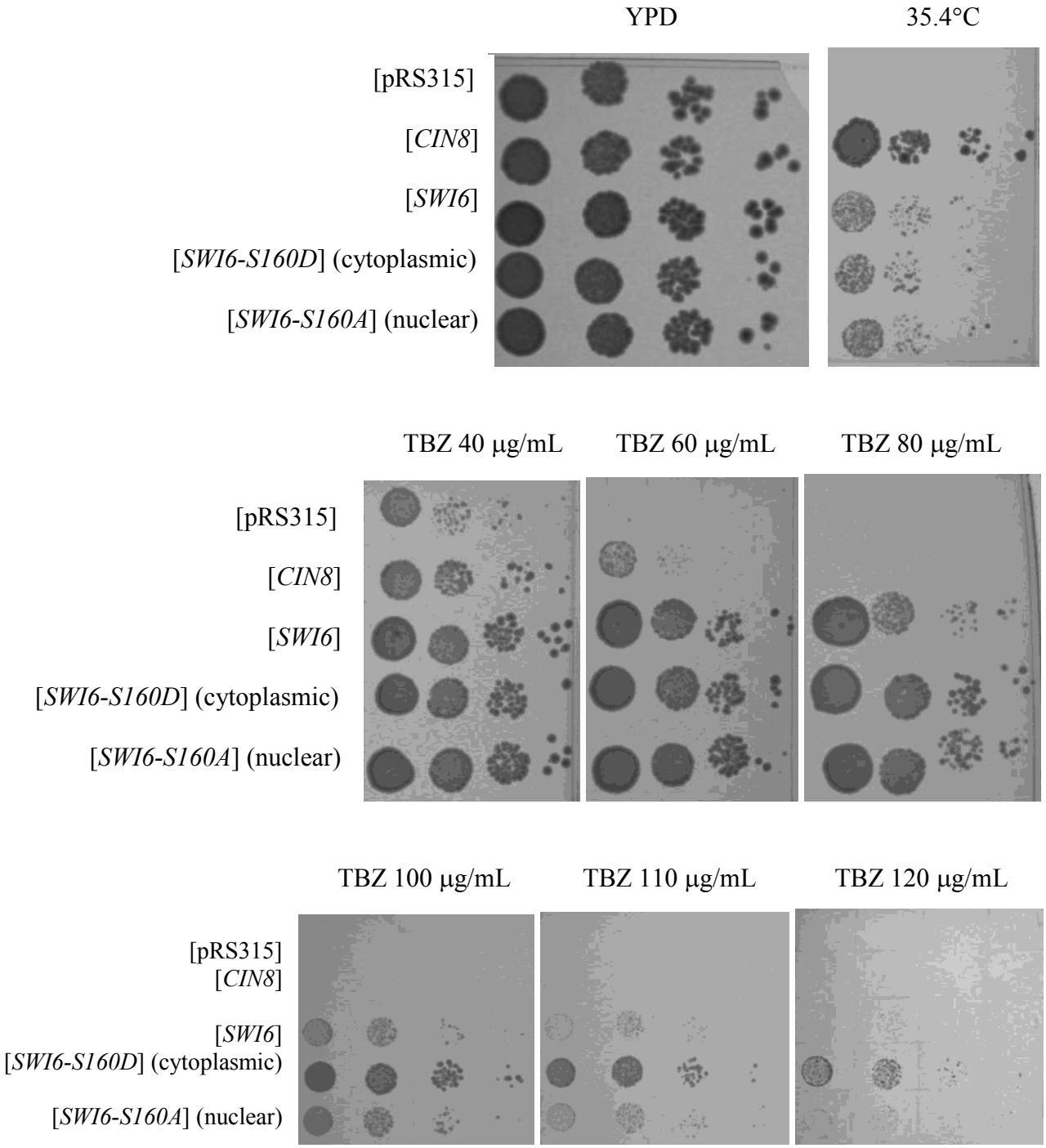
We also examined the protein's localization in triple mutants of *cin8-3 kip1Δ swi4Δ, cin8-3 kip1Δ clb2Δ, cin8-3 kip1Δ hsl1Δ,* and *cin8-3 kip1Δ elm1Δ* but saw no difference in Swi6-mCherry's cell-cycle localization (not shown). These results suggest that the role of *FCP1* overexpression and the role of the other genes that came up in our screen in the suppression does not stem from altering the cellular localization of Swi6.

A role for specific alleles of *SWI6* in alleviating the defect associated with *cin8-3, kip1Δ* cells

To complement these studies, we obtained nuclear (Swi6-S160A) and predominantly cytoplasmic (Swi6-S160D) alleles of *SWI6* and introduced them individually into the triple mutant *cin8-3 kip1Δ swi6Δ* overexpressing *FCP1*. We tested for complementation of growth at the non-permissive temperature and under MT-destabilizing conditions. As shown in Figure 4.8, introduction of a wild-type copy of *SWI6* on a centromeric plasmid resulted in partial complementation of the temperature-sensitive phenotype, as did the other alleles of the gene. All three alleles, however, conferred high levels of resistance to the microtubule-destabilizing drug TBZ.

Interestingly, the predominantly cytoplasmic version of *SWI6* (Swi6-S160D) demonstrated hyperstability to TBZ. Since expression of neither allele proved to alter SBF-driven transcription[145], the following explanations may be offered to account for these observations: Swi6 may have a cytoplasmic role that is relevant for the suppression, possibly involving interaction with cytoplasmic proteins leading to MT stability (i.e., through cross-talk between cytoplasmic and nuclear machineries, see discussion in Chapter V). The other possibility is that the S160D mutation, independent of altering Swi6p's cellular localization, confers some novel biochemical properties to the protein that allow it to stabilize MTs in our background under the conditions assayed.

Figure 4.8 Serial dilutions of *cin8-3 kip1Δ swi6 Δ [FCP1-2μ]* cells containing Centromeric plasmids indicated on the left:



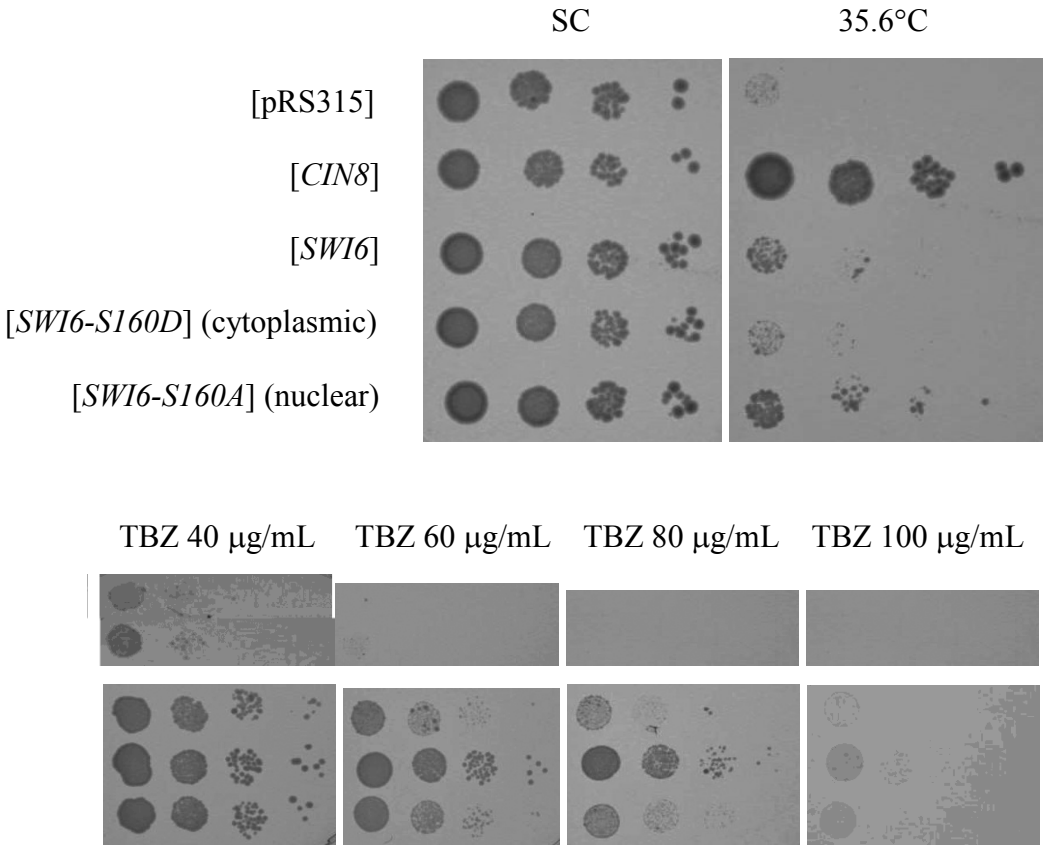
Since overexpression of *FCP1* in *cin8-3 kip1Δ* cells does not alter the cellular localization of Swi6-mCherry (as assayed by fluorescence microscopy) even though expression of the predominantly cytoplasmic allele of *SWI6* results in TBZ resistance that actually exceeds that of *cin8-3 kip1Δ* overexpressing *FCP1* (not shown), this presents one of two possibilities. The first is that what is significant for the suppression in our background is not Swi6p's localization but the *SWI6*(S160D) allele, which may introduce a novel function to the protein (other than merely altering it cellular distribution), such as a function concerned with MT stability. Another possibility is that the suppression catered by expression of the cytoplasmic version of Swi6 acts in a different pathway to MT stability than *FCP1* overexpression. In other words, cytoplasmic Swi6 specifically (as opposed to wild-type *SWI6*) does not mediate *FCP1*'s effect on MT stability, nonetheless, it may substitute for *FCP1* overexpression when MT stability is deficient.

To provide further evidence for this latter premise, we tested whether any of the alleles of *SWI6* could suppress the temperature- and TBZ sensitivity in the absence of *FCP1* overexpression. To achieve this, triple mutants were streaked on YPD+geneticin plates from 5 to 15 times to ensure the loss of the multicopy plasmid carrying *FCP1* and cells were checked to see that they indeed lost the ability to grow at the non-permissive temperature. Figure 4.8 reveals that while plasmids carrying the wild-type or the nuclear version of *SWI6* no longer supported growth at TBZ concentrations that compromised the double mutant, the predominantly cytoplasmic version of Swi6, although less resilient at extremely high TBZ concentrations in the absence of *FCP1* overexpression, is nonetheless able to restore growth to the level of the double-mutant overexpressing *FCP1*

(see Figure 4.9). This suggests that the effect of the cytoplasmic allele on MT stability is independent of *FCP1* overexpression and may substitute for it.

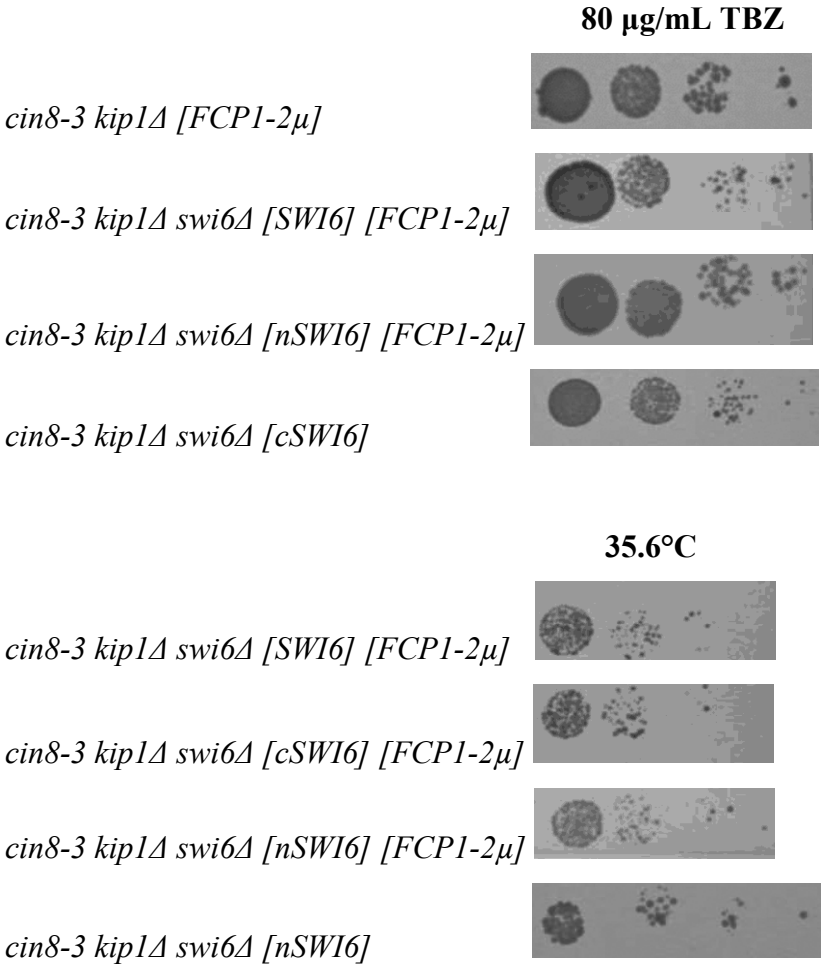
Furthermore, when comparing the different concentrations of TBZ that produce the same levels of growth in the triple mutant *cin8-3 kip Δ swi6Δ* [*SWI6-S160D*] with (Figure 4.8) or without (Figure 4.9) *FCP1* overexpression, it seems that *FCP1* enhances the effect of the predominantly cytoplasmic allele of *SWI6*, producing hyper-resistance to MT-destabilizing conditions. Their effects on MT stability appear to be additive and therefore may be part of different pathways. Finally, in the absence of *FCP1* overexpression, only the constitutively nuclear allele of *SWI6* is able to suppress the temperature-sensitivity of our mutants.

Figure 4.9 Serial dilutions of *cin8-3 kip1Δ swi6 Δ* cells containing the centromeric plasmids indicated on the left:



Figures 4.10 below compares the restoration of growth of the triple mutant *cin8-3 kip1Δ swi6Δ* by the different alleles of *SWI6* under selected conditions.

Figure 4.10 Plasmids required in the double mutant *cin8-3 kip1Δ* to match the level of suppression catered by *FCP1* overexpression. Suppression of a.) TBZ sensitivity; and b.) temperature sensitivity. Lower case *c* for predominantly cytoplasmic or S160D allele; lower case *n* for nuclear or S160A allele



We may therefore conclude that *FCP1* overexpression, in collaboration with *SWI6* or the different alleles tested, may restore growth at the nonpermissive temperature and restrictive TBZ concentrations. In addition, the specific alleles may, independently of *FCP1* overexpression, achieve the same level of suppression in the double mutant. However, the suppressive potential of the S160D/cytoplasmic allele was pronounced when the mutants were grown on TBZ plates, whereas that of the S160A/nuclear allele was prominent in the temperature-sensitivity experiments.

Based on the results presented in this chapter, we propose that the synergistic enhancement of the TBZ sensitivity observed in triple mutants of *cin8-3 kip1Δ swi6Δ* is due to a dual role of Swi6 in MT stability that may be working in parallel: one that is *FCP1* overexpression-dependent and one that is *FCP1* overexpression-independent. The *FCP1* overexpression-dependent "pathway" works via Swi4, by virtue of its interaction with it and their cooperative involvement in the SBF complex. This is supported by the finding that *FCP1* overexpression correlates with a proportional reduction in the levels of Swi4 and Swi6 proteins by the same factor, suggestive of complex involvement (See Figure 4.2). This may result in re-routing of the SBF complex to selected promoters, possibly of genes that contribute to MT stability.

The other pathway is *FCP1* overexpression-independent. This pathway is also independent of the SBF complex, as neither the S160D/cytoplasmic nor the S160A/nuclear alleles of *SWI6* were shown to be alter SBF-mediated transcription[145]. This pathway is dependent on the phosphorylation and/or localization state of Swi6p. Determination of the preferred phosphorylation/localization state seems to depend on the assay we design to test it: MTs affected by growth at the non-permissive temperatures are intranuclear MTs, as both Cin8 and Kip1 which are mutated in our background bind interpolar MTs of the spindle midzone (see Chapter I and Chapter III). It is not surprising then that if Swi6 has a role in MT stability, then its presence in the nucleus would stabilize nuclear MTs. Growing our cells in media supplemented with the MT-destabilizing drug TBZ may affect the overall state of both nuclear and cytoplasmic MTs, and therefore, by expressing the S160D allele we are effectively keeping Swi6p in

potential contact with both nuclear and cytoplasmic MTs, as the allele localizes to both compartments (though it is predominantly nuclear).

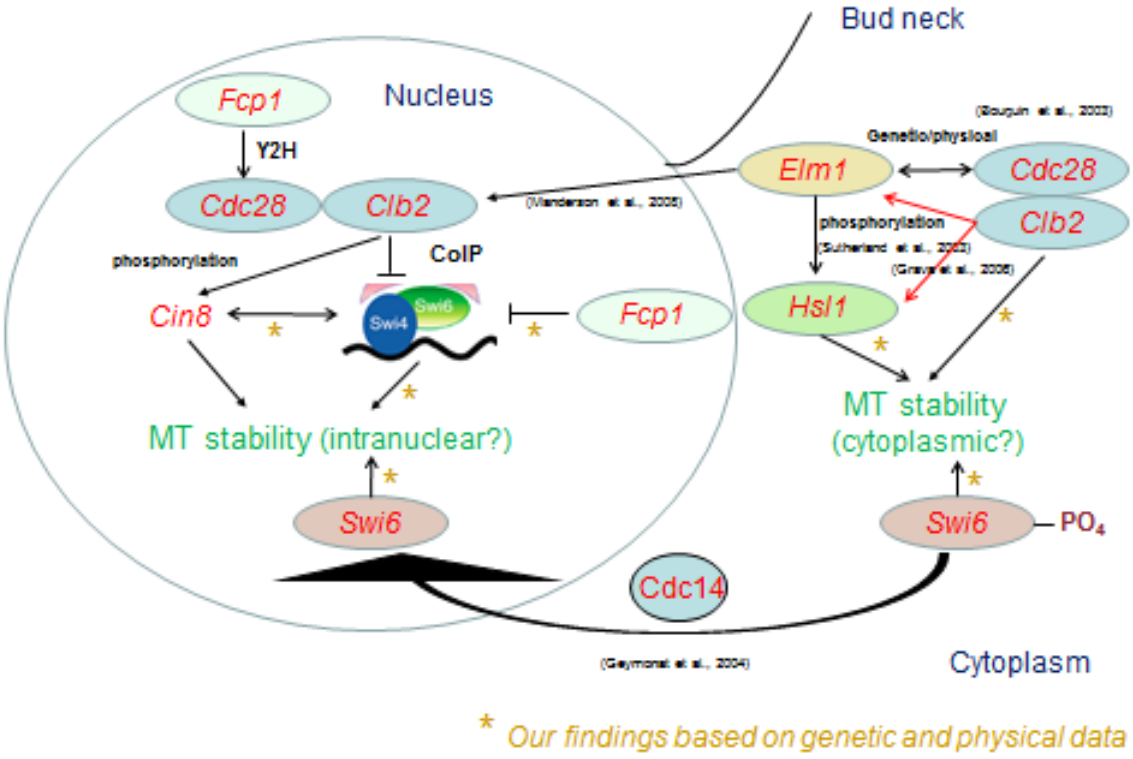
Conclusion

In Chapter III and IV we discovered genes that may have a role in regulating MT stability as their deletion in the *cin8-3 kip1Δ* background abrogates the suppression catered by *FCP1* overexpresssion. Further work should be done to establish roles for specific alleles or map specific regions of the protein that are necessary for the suppression, and once found, to examine whether these elements can restore the wild-type phenotype in the absence of *FCP1* overexpression. This was done only for *SWI6* as we had access to two cell-cycle specific alleles. The most significant contribution of this chapter is probably the finding that these specific alleles of *SWI6* can alleviate the temperature and TBZ-sensitivity of our mutants independently of *FCP1* overexpression.

Interestingly, the phosphatase activity of the principal mitotic exit regulator Cdc14p was shown to be required for nuclear microtubule stabilization at anaphase onset when the spindle starts to elongate[42]. The same phosphatase was shown to be able to trigger nuclear import of Swi6p *in vivo* and to dephosphorylate Swi6p at serine 160 *in vitro*[112]. This presents the possibility that Cdc14 may mediate MT stability when the spindle starts to elongate (a time when our motor mutants arrest at elevated temperatures, and when Clb2p levels peak, see Chapter III) by recruiting Swi6p into the nucleus. This is supported by our findings that that the nuclear version of Swi6p restores growth at the non-permissive temperature independently of *FCP1* overexpression.

Based on the results presented in this and the previous chapter the following model may be constructed (see Figure 4.11). Double arrows between Cin8, Kip1 and Swi4 represent uncertainty with regards to the genes' epistatic order based on our results. As in Figure 3.17, we cannot exclude the participation of unidentified components in the scheme proposed below. A thorough discussion of the pathways drawn is presented in the next chapter.

Figure 4.11 A model of genetic and physical interactions. Based on results presented in this and the previous chapter and in the literature



CHAPTER V

DISCUSSION

5.1 PUTATIVE ROLE OF *FCP1* IN THE SUPPRESSION

Fcp1 specifically dephosphorylates the C-terminal domain of RNA polymerase II (see Chapter II). The significance of RNAPII CTD phosphorylation is iterated in the proposal of a "CTD code"[148] that conveys information to CTD-binding proteins, some of which recognize particular phosphorylation patterns and trigger specific cellular responses[149]. Fcp1p is a key player in shaping such patterns, it is essential in budding and fission yeast, and is conserved among eukarya[84]. In this work we demonstrated that the phosphatase activity of Fcp1p is necessary for the suppression of the temperature-sensitivity of our mutants and that the overexpression of structural and functional homologues of the protein do not produce the same effect. Furthermore, replacing the catalytic aspartate residues of the conserved LVVDLDQTII peptide motif was previously shown to effectively knock out the CTD phosphatase activity of the yeast Fcp1p[66], and in our studies, these key residues were found to be essential for the suppression of the temperature sensitivity of our mutants. Moreover, we showed that deleting a region that interacts with TFIIF and TFIIB abolishes the suppression.

How can overexpression of Fcp1's phosphatase activity then, directed specifically to the transcriptional process, suppress defects associated with a compromised microtubule cytoskeleton?

Mutations in *FCP1* were shown to result in increased phosphorylation of Ser2 and rapid shutdown of mRNA synthesis by RNAPII[75, 150]. Conversely, *FCP1* overexpression in *S. pombe* was shown to result in an increase in the level of the hypophosphorylated (IIa) form of Rbp1 relative to control strains[151]. Similarly, by overexpressing *FCP1* in our strains we may be mimicking massive dephosphorylation of the CTD of RNA

polymerase II. It was demonstrated in *Xenopus* extracts that upon fertilization, this C-terminal domain undergoes fast and massive dephosphorylation that is attributable to the *Xenopous* orthologue of *FCP1*[152]. This dephosphorylation contributes to the preparation of the transcriptional machinery for zygotic genome activation, which is accompanied by a shift between dramatically different gene expression programs for the fertilized oocyte.

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[153]. Similarly, *FCP1* overexression in our genetic background may specify a transcriptional program that is accompanied by changes in MT stability.

5.2 PUTATIVE ROLE FOR BUD NECK-LOCALIZED PROTEINS IN THE SUPPRESSION

The finding that deletions of *CLB2*, *ELM1*, and *HSL1* all abrogate the suppression of *FCP1* overexpression is significant not only in the context of the individual genetic, spatial, and temporal connections established in Chapter III, but equally important, in light of their implication of cytoplamic MTs and the bud neck in transmitting MT stabilizing cues to the nucleus. This putative role for the proteins is somewhat enigmatic for both our model system and the genetic background we employ: *S. cervisiae* cells exhibit closed mitosis in which the nuclear envelope remains intact, forming a barrier between nuclear and cytoplasmic MTs, and in our genetic background it is the

intranuclear microtubule cytoskeleton is compromised due to defects of motors that localize to interpolar MTs of the spindle midzone. Cross-talk between the two kinds of MTs or compartments is therefore expected to be rather complicated.

In higher eucaryotes cross-talk between the nucleus and the cytoplasm is simpler. Higher eukaryotes display open mitosis in which the nuclear envelope is completely disassembled in prometaphase and is not reassembled until after DNA segregation in telophase/G_1, providing an opportunity for "mixing" of nuclear and cytoplasmic proteins, thereby facilitating communication. In fact, in such a system the spindle midzone and proteins associated with it were shown to be essential for proper cytokinesis[154], a cortical event. Specifically, evidence was presented that chromosomal passenger proteins couple spindle formation and stability with positioning at the site of cytokinesis. These passenger proteins associate with chromosomes along their length during prophase, becoming concentrated at the inner centromere by metaphase, and upon transition to anaphase the passengers abruptly transfer to the central region of the mitotic spindle and to the cell cortex in a region where the cleavage furrow will form[154].

Furthermore, Canman et al.[155] demonstrated that after anaphase onset but before furrow onset there is a marked polymerization of MTs toward the cell cortex in the region of future furrow formation in mammalian tissues, suggesting direct contact between numerous stable, chromosome-associated MT ends and the cell cortex. This and other evidence led the authors to propose that chromosome- associated (kinetochore) MTs form persistent associations with the cell cortex through transport of centromere-bound MT stabilization factors and/or passengers to the plus ends of these MTs, stimulating formation of a productive cleavage furrow at the cell equator. In *Drosophila* and *C.*

elegans embryos, motors were shown to play a role in cytokinesis by virtue of their localization to the spindle midzone[154].

In yeast, closed mitosis is the *modus operandi* so envisioning a direct involvement for the inner spindle in specification of cytokinesis seems less likely. Still, there is evidence in yeast to support a coordination of spindle stability during anaphase with cytokinesis, though the mechanisms have not been elucidated. For instance, in budding yeast, a protein that was suggested to play a role in such coordination is the chromosomal passenger protein Ndc10p. Ndc10p localizes from the inner kinetochore to the spindle midzone and was shown to play a role in maintaining spindle stability during anaphase. In addition to defects in anaphase spindle elongation, *ndc10-1* cells fail to properly organize septins[156].

In fission yeast, sister chromatid separation was shown to be delayed in *cdc11-123* cells, mutants in which nucleation of astral MTs is partially disrupted by septin defects[157]. Also in fission yeast, a deletion of *MTO1*, a gene required for normal cytoplasmic MT organization, was shown to lead to defects in centromere clustering[158]. Furthermore, Tournier *et al.*[159] demonstrated in the same yeast that sister kinetochore congression at the spindle midzone before anaphase onset is disturbed when astral MT contact with the cell cortex is disrupted. They also showed that the kinetochore-localized checkpoint protein kinase Bub1 can impose a delay in the timing of chromosome separation at the kinetochores when astral MT interaction with the cell cortex is disturbed[157]. The authors propose that a signal must be transmitted from the cell cortex to the kinetochore for nuclear Bub1 to activate the spindle-orientation checkpoint.

In these instances, the proteins localize to one cellular compartment, yet their associated phenotype suggests that they have a role in a compartment they are not known to be situated in. This presents the possibility that these proteins affect events at the other compartment indirectly to relay the message. Proposing that a stabilizing signal is somehow conveyed from astral MTs contacting the bud neck to intranuclear MTs requires a mechanism by which the signal is transduced from the cortex to the midzone. There are several ways by which this could be enabled in cells. The first is biophysical in nature: asymmetric tension forces may be transmitted from astral MTs contacting the cortex to intranuclear microtubules. These forces may transverse the plaques of the nuclear envelope in which the SPB is embedded in, communicating tension. Proteins embedded in the nuclear envelope may aid in relaying such vectorial information. In fission yeast, for example, cytoplasmic MTs were shown to be mechanically coupled to nuclear heterochromatin through integral membrane proteins embedded in the nuclear envelope in regions of the MTOC attachment site of the SPB, the structure to which force and/or tension are transmitted[160]. These tethering proteins were also shown to be responsible for withstanding forces exerted by astral MTs on the nucleus, forces which drive its movement during spindle positioning. It was suggested that such "buffering" of forces is responsible for maintaining the integrity of the nuclear envelope as the nucleus is propelled through the cytoplasm. Furthermore, the ramifications of heterochromatin clustering adjacent to the site of SPB attachment is that it presents the opportunity for centromeric proteins to affect astral MT dynamics and stability, and this is supported by the finding that the specific heterochromatic state (which is characterized by a particular milieu of proteins) affect SPB functioning.

The findings that demonstrate that molecular linkages, that are independent of the nuclear pore complex, exist between the nuclear interior and the cytoplasmic cytoskeleton, provide a means for communication between the cytoplasm and the nucleus that is and present paradigms for astral MTs affecting intranuclear structures.

In budding yeast, a role for nuclear envelope proteins in coupling nuclear and cytoplasmic cytoskeletal events was suggested to involve the nuclear cyclin Clb5p. Clb5 was shown to be required for both spindle assembly and orientation, as its loss abolishes the intrinsic asymmetry between spindle poles, which is required for translocation of the spindle into the bud[161]. The authors showed that the protein's genetic interaction with Spc110, the nuclear envelope inner plaque receptor for the γ-tubulin complex (which nucleates intranuclear MTs), is involved in restoring this polarity defect.

Other than communicating via the nuclear envelope, proteins may transmit tension information or convey positional information (i.e., about the coordinates and orientation of the mitotic spindle in relation to the site of cytokinesis) between the different cellular compartments by shuttling in and out of the nucleus. Such proteins would be expected to be localized to both intranuclear MTs and astral MTs (either at their plus end contacting the cortex, throughout the lattice, or at their minus end close to SPB that nucleates them). Good candidates would be checkpoint proteins or their regulators that mediate crosstalk between the spindle assembly and the spindle position checkpoint. Therefore, these proteins may be necessary for coupling anaphase completion with cytokinesis. For example, fission yeast Ase1, a MAP and a regulatory component in the cytokinesis checkpoint, localizes to both the spindle midzone and SPBs during mitosis, and along anaphase astral MTs. This disposition allows it to inhibit nuclear division when

the cytokinesis apparatus is perturbed. *ase1* deletion manifests in an abrupt collapse of elongating anaphase B spindles, as well as in defects in nuclear and septum positioning and completion of cytokinesis[162]. Furthermore, in budding yeast, checkpoint proteins Bub2 and Bfa1 were shown to participate in both spindle assembly and the spindle position checkpoints[136].

In addition to communicating MT stability by shuttling MAPs or potential MAP regulators between the two compartments, tubulin flux between the nucleus and the cytoplasm may account for changes in MT stability. Souza and Osmani[163]suggested that in the budding yeast closed mitosis was able to evolve largely because the spindle pole bodies that are embedded in the nuclear envelope can nucleate microtubules from *either* their cytoplasmic or nuclear face. It is therefore not essential to break down the nuclear envelope in order to form a mitotic spindle. Rather, spindle pole bodies need only to change their site of microtubule nucleation from the cytoplasmic face during interphase to the nuclear face during mitosis. One way to regulate this is to restrict when tubulin can enter the nucleus during the cell cycle, and in fact, the authors report that in *S. cerevisiae* nuclear levels of tubulin (and levels of other mitotic regulators) increases gradually as the spindle elongates during the cell cycle. The authors suggest that a mechanism may exist which fine tunes nuclear uptake of tubulin by modifying mitosis-specific active transport to the nucleus.

In agreement with this idea, there are several lines of evidence for a shift of MT density from the nucleus to the cytoplasm in yeast. Strains with a deletion of *SLK19* were shown to exhibit abnormally short mitotic spindles accompanied by increased numbers of astral MTs[164]. This phenotype was similar to that of *kar3* mutants, except that the latter

also exhibited increased length of cytoplasmic MTs[48, 164]. In *slk19Δ kar3Δ* double mutants, these phenotypes were accentuated: short spindles collapsed causing the two SPBs to converge, followed by an increase in cytoplasmic MT number and length. Neither of these proteins has been shown to localize to the cytoplasm or cytoplasmic MTs: Slk19p localizes to centromeres and the spindle midzone, where it is proposed to stabilize MTs at the midzone and maintain pre-anaphase spindle bipolarity. Kar3p was shown to localize to the nuclear face of the SPB. The authors propose that it is possible that the growth of cytoplasmic MTs may be an indirect consequence of the depolymerization of nuclear MTs that make up the spindle.

Can any of these models of communication between the cytoplasmic and nuclear cytoskeleton apply to MT stability in our system? It was recently shown[165] that in a system in which spindle elongation is prevented, a *cin8* deletion leads to defects in SPB separation accompanied by an elevated number of astral MTs and a change in their organization within the cell resulting in a "daugterly phenotype". The authors suggest that excess MTs on the daughter side could lead to a disproportionate pulling force toward the daughter cell, resulting in nuclear positioning defects, with DNA being segregated predominantly in the daughter. Furthermore, the authors observed that when the spindle is allowed to form and SPBs are able to separate in these mutants, the number of astral MTs was significantly lower than in cells that failed to separate their SPBs. Because this failure to separate the SPBs correlated with an increase in astral MT number, the authors proposed that the increase in astral MTs that accompanies these mutants is the result of excess tubulin (due to the absence of a spindle) that is available to polymerize outside of the nucleus in the form of cytoplasmic MTs.

According to this paradigm, if *FCP1* overexpression in our mutants is able to restore the MT stability necessary for accomplishing spindle assembly and elongation, then a deletion in the septin-dependent kinase Hsl1 would compete with the suppression by leading to excessive stabilization of MT binding at the bud neck, as reported by Kusch et al. [128]. In other words, the steady-state number of MTs attached at the bud neck would increase at the expense of the nuclear MTs of the mitotic spindle. Therefore, Hsl1 and the other bud neck-localized proteins that were revealed in our screen and genetically interact with or regulate Hsl1 activity may ultimately ensure that MT interactions with the bud neck remain dynamic so that the cell could effectively respond to tubulin demand in deficient regions, such as in the nuclear spindle.

5.3 NOVEL FUNCTIONS IN MICROTUBULE STABILITY

The genes that came up in our screen can be divided into two groups: genes that are in the same putative pathway as *FCP1* to MT stability and therefore their deletion essentially cuts off this vital "lifeline" to MT stability, and genes that work in a pathway parallel to but that converges at a node with *FCP1* overexpression. The question that inevitably arises is why, under conditions in which *FCP1* is not overexpressed, do the wild-type genes that came up in our screen not reveal their MT-stabilizing potential? The answer may lie in an evolutionary phenomenon known as genetic buffering. Genetic buffering is a process that suppresses selected phenotypes associated with a protein from being expressed. It is often caused by other genes that redundantly function in the same process, whether they belong to the same or convergent pathways[166].

S. Rutherford[167] explained that in wild-type cells, a large amount of silent, but potentially very potent functional variation is maintained. Genetic buffering limits functional polymorphism, controlling which proteins can express any given phenotype that's associated with them. Stressful conditions break down this buffering. The result is that thresholds governing the expression of previously dormant functions are lowered, such that the activity level required to usher in a phenotypic consequence is more easily met. At these thresholds, phenotypic differences suddenly appear. Relating this to our results, when any of the genes that came up in our secondary screen are deleted from the motor mutant background, a presumably novel phenotype associated with a deletion of these genes emerges, though it may have only been concealed in the wild-type. Under MT-destabilizing conditions such as elevated temperatures or growth on media containing drugs that interfere with MT polymerization, buffering may break down so that the phenotype associated with these genes is no longer suppressed, assigning a previously uncharacterized role for the proteins.

The mechanism of genetic buffering acting on Swi6 may be the transient expression of different phosphorylated forms and their restriction to specific cell-cycle phases. Constitutive expression of these alleles in our background under MT-destabilizing conditions serves to relieve or disrupt constraints set by genetic buffering, allowing Swi6's functional potential, namely, its role in MT stability, to manifest phenotypically. Furthermore, the demonstration that specific variants of *SWI6* can support MT stability in our background independently of *FCP1* overexpression may be identified with the process of canalization, which is characterized by resilience of normal functioning in the face of environmental perturbations or genetic variation, leading to the

attainment of the same endpoint, even if by an alternate route. An altered genetic circuitry was reported in instances in which parallel pathways feed into the same process[167].

An example from the literature of how the connectivity of signaling pathways can change while the phenotype remains constant is taken from MAP kinase signaling pathways in yeast. Some proteins in the MAP kinase signaling cascades are shared with other signal transduction systems, for example, the pseudohyphal growth pathway shares a number of components with two other signal transduction systems, those sensing osmotic stress and those sensing the presence of the mating pheromone. A null mutant of Kss1, a protein now known to be a component of the mating pathway, has no phenotypic consequence on the pathway, so it was originally thought not to be involved in the process. It was later shown that it can fill in for another protein, Fus3, which is functionally redundant with it, when it is not present[167]. Similarly, Swi6 has no known role in MT stability in wild-type cells, yet our results suggest that Swi6 may, in addition to its role in the regulation of transcription at the G_1/S boundary, participate in the regulation of MT stability in *cin8-3 kip1Δ* cells, possibly altering the strain's default genetic circuitry.

SUMMARY

Regulation of MT stability is critical for mitotic events. Our work assigned novel roles in MT stability for proteins that were not previously linked to the process or that had phenotypes minimally suggestive of MT involvent. These proteins may be candidate MAPs or novel regulators of MAPs (whether direct or indirect). Genetic interactions, epistatic experiments, and physical data (with additions from the literature) led us to

propose a pathway to MT stability, outlined in Figure 4.11. More studies should be done to conclusively establish the connections presented in our model. This knowledge may enhance the understanding of MT regulation that may have therapeutic implications for the treatment of ailments such as cancer.

REFERENCES

1. Bouck DC, Joglekar AP, Bloom KS: Design features of a mitotic spindle: balancing tension and compression at a single microtubule kinetochore interface in budding yeast, Annu Rev Genet 2008, 42:335-359

2. Hyman AA, Karsenti E: Morphogenetic properties of microtubules and mitotic spindle assembly, Cell 1996, 84:401-410

3. Nogales E, Whittaker M, Milligan RA, Downing KH: High-resolution model of the microtubule, Cell 1999, 96:79-88

4. Sorger PK, Dobles M, Tournebize R, Hyman AA: Coupling cell division and cell death to microtubule dynamics, Curr Opin Cell Biol 1997, 9:807-814

5. Caplow M, Shanks J: Evidence that a single monolayer tubulin-GTP cap is both necessary and sufficient to stabilize microtubules, Mol Biol Cell 1996, 7:663-675

6. Mandelkow EM, Mandelkow E, Milligan RA: Microtubule dynamics and microtubule caps: a time-resolved cryo-electron microscopy study, J Cell Biol 1991, 114:977-991

7. Caplow M, Ruhlen RL, Shanks J: The free energy for hydrolysis of a microtubule-bound nucleotide triphosphate is near zero: all of the free energy for hydrolysis is stored in the microtubule lattice, J Cell Biol 1994, 127:779-788

8. Alberts B, Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P.: Molecular Biology Of The Cell. Edited by 2006, p.

9. Byers B: Cytology of the Yeast Life Cycle. Edited by J.N. Strathern EWJ, and J.R. Broach. Cold Spring Harbor Laboratory

1981, p. pp. 59-96

10. Huffaker TC, Thomas JH, Botstein D: Diverse effects of beta-tubulin mutations on microtubule formation and function, J Cell Biol 1988, 106:1997-2010

11. Jacobs CW, Adams AE, Szaniszlo PJ, Pringle JR: Functions of microtubules in the Saccharomyces cerevisiae cell cycle, J Cell Biol 1988, 107:1409-1426

12. Barnes G, Louie KA, Botstein D: Yeast proteins associated with microtubules in vitro and in vivo, Mol Biol Cell 1992, 3:29-47

13. Olmsted JB: Microtubule-associated proteins, Annu Rev Cell Biol 1986, 2:421-457

14. Joshi HC: Microtubule dynamics in living cells, Curr Opin Cell Biol 1998, 10:35-44

15. DeLuca JG, Gall WE, Ciferri C, Cimini D, Musacchio A, Salmon ED: Kinetochore microtubule dynamics and attachment stability are regulated by Hec1, Cell 2006, 127:969-982

16. Lin H, de Carvalho P, Kho D, Tai CY, Pierre P, Fink GR, Pellman D: Polyploids require Bik1 for kinetochore-microtubule attachment, J Cell Biol 2001, 155:1173-1184

17. Berlin V, Styles CA, Fink GR: BIK1, a protein required for microtubule function during mating and mitosis in Saccharomyces cerevisiae, colocalizes with tubulin, J Cell Biol 1990, 111:2573-2586

18. Tirnauer JS, O'Toole E, Berrueta L, Bierer BE, Pellman D: Yeast Bim1p promotes the G1-specific dynamics of microtubules, J Cell Biol 1999, 145:993-1007

19. Janson ME, Loughlin R, Loiodice I, Fu C, Brunner D, Nedelec FJ, Tran PT: Crosslinkers and motors organize dynamic microtubules to form stable bipolar arrays in fission yeast, Cell 2007, 128:357-368

20. Rout MP, Kilmartin JV: Components of the yeast spindle and spindle pole body, J Cell Biol 1990, 111:1913-1927

21. Spang A, Geissler S, Grein K, Schiebel E: gamma-Tubulin-like Tub4p of Saccharomyces cerevisiae is associated with the spindle pole body substructures that organize microtubules and is required for mitotic spindle formation, J Cell Biol 1996, 134:429-441

22. O'Toole ET, Winey M, McIntosh JR: High-voltage electron tomography of spindle pole bodies and early mitotic spindles in the yeast Saccharomyces cerevisiae, Mol Biol Cell 1999, 10:2017-2031

23. Chretien D, Fuller SD, Karsenti E: Structure of growing microtubule ends: two-dimensional sheets close into tubes at variable rates, J Cell Biol 1995, 129:1311-1328

24. Shaw SL, Yeh E, Maddox P, Salmon ED, Bloom K: Astral microtubule dynamics in yeast: a microtubule-based searching mechanism for spindle orientation and nuclear migration into the bud, J Cell Biol 1997, 139:985-994

25. Lee L, Tirnauer JS, Li J, Schuyler SC, Liu JY, Pellman D: Positioning of the mitotic spindle by a cortical-microtubule capture mechanism, Science 2000, 287:2260-2262

26. Sablin EP: Kinesins and microtubules: their structures and motor mechanisms, Curr Opin Cell Biol 2000, 12:35-41

27. Karki S, Holzbaur EL: Cytoplasmic dynein and dynactin in cell division and intracellular transport, Curr Opin Cell Biol 1999, 11:45-53

28. Hildebrandt ER, Hoyt MA: Mitotic motors in Saccharomyces cerevisiae, Biochim Biophys Acta 2000, 1496:99-116

29. Eshel D, Urrestarazu LA, Vissers S, Jauniaux JC, van Vliet-Reedijk JC, Planta RJ, Gibbons IR: Cytoplasmic dynein is required for normal nuclear segregation in yeast, Proc Natl Acad Sci U S A 1993, 90:11172-11176

30. Lillie SH, Brown SS: Immunofluorescence localization of the unconventional myosin, Myo2p, and the putative kinesin-related protein, Smy1p, to the same regions of polarized growth in Saccharomyces cerevisiae, J Cell Biol 1994, 125:825-842

31. Sawin KE, LeGuellec K, Philippe M, Mitchison TJ: Mitotic spindle organization by a plus-end-directed microtubule motor, Nature 1992, 359:540-543

32. Hoyt MA, He L, Loo KK, Saunders WS: Two Saccharomyces cerevisiae kinesin-related gene products required for mitotic spindle assembly, J Cell Biol 1992, 118:109-120

33. Saunders WS, Hoyt MA: Kinesin-related proteins required for structural integrity of the mitotic spindle, Cell 1992, 70:451-458

34. Saunders WS, Koshland D, Eshel D, Gibbons IR, Hoyt MA: Saccharomyces cerevisiae kinesin- and dynein-related proteins required for anaphase chromosome segregation, J Cell Biol 1995, 128:617-624

35. Cottingham FR, Hoyt MA: Mitotic spindle positioning in Saccharomyces cerevisiae is accomplished by antagonistically acting microtubule motor proteins, J Cell Biol 1997, 138:1041-1053

36. Segal M, Clarke DJ, Reed SI: Clb5-associated kinase activity is required early in the spindle pathway for correct preanaphase nuclear positioning in Saccharomyces cerevisiae, J Cell Biol 1998, 143:135-145

37. Lew DJ: Cell-cycle checkpoints that ensure coordination between nuclear and cytoplasmic events in Saccharomyces cerevisiae, Curr Opin Genet Dev 2000, 10:47-53

38. Yeh E, Skibbens RV, Cheng JW, Salmon ED, Bloom K: Spindle dynamics and cell cycle regulation of dynein in the budding yeast, Saccharomyces cerevisiae, J Cell Biol 1995, 130:687-700

39. DeZwaan TM, Ellingson E, Pellman D, Roof DM: Kinesin-related KIP3 of Saccharomyces cerevisiae is required for a distinct step in nuclear migration, J Cell Biol 1997, 138:1023-1040

40. Carminati JL, Stearns T: Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex, J Cell Biol 1997, 138:629-641

41. Winey M, Mamay CL, O'Toole ET, Mastronarde DN, Giddings TH, Jr., McDonald KL, McIntosh JR: Three-dimensional ultrastructural analysis of the Saccharomyces cerevisiae mitotic spindle, J Cell Biol 1995, 129:1601-1615

42. Higuchi T, Uhlmann F: Stabilization of microtubule dynamics at anaphase onset promotes chromosome segregation, Nature 2005, 433:171-176

43. Peterson JB, Ris H: Electron-microscopic study of the spindle and chromosome movement in the yeast Saccharomyces cerevisiae, J Cell Sci 1976, 22:219-242

44. Roof DM, Meluh PB, Rose MD: Multiple kinesin-related proteins in yeast mitosis, Cold Spring Harb Symp Quant Biol 1991, 56:693-703

45. Madden K, Costigan C, Snyder M: Cell polarity and morphogenesis in Saccharomyces cerevisiae, Trends Cell Biol 1992, 2:22-29

46. McNally FJ: Modulation of microtubule dynamics during the cell cycle, Curr Opin Cell Biol 1996, 8:23-29

47. Endow SA, Kang SJ, Satterwhite LL, Rose MD, Skeen VP, Salmon ED: Yeast Kar3 is a minus-end microtubule motor protein that destabilizes microtubules preferentially at the minus ends, EMBO J 1994, 13:2708-2713

48. Saunders W, Hornack D, Lengyel V, Deng C: The Saccharomyces cerevisiae kinesin-related motor Kar3p acts at preanaphase spindle poles to limit the number and length of cytoplasmic microtubules, J Cell Biol 1997, 137:417-431

49. Huyett A, Kahana J, Silver P, Zeng X, Saunders WS: The Kar3p and Kip2p motors function antagonistically at the spindle poles to influence cytoplasmic microtubule numbers, J Cell Sci 1998, 111 (Pt 3):295-301

50. Cottingham FR, Gheber L, Miller DL, Hoyt MA: Novel roles for saccharomyces cerevisiae mitotic spindle motors, J Cell Biol 1999, 147:335-350

51. Saunders W, Lengyel V, Hoyt MA: Mitotic spindle function in Saccharomyces cerevisiae requires a balance between different types of kinesin-related motors, Mol Biol Cell 1997, 8:1025-1033

52. Belmont LD, Hyman AA, Sawin KE, Mitchison TJ: Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts, Cell 1990, 62:579-589

53. Richardson HE, Wittenberg C, Cross F, Reed SI: An essential G1 function for cyclin-like proteins in yeast, Cell 1989, 59:1127-1133

54. Fitch I, Dahmann C, Surana U, Amon A, Nasmyth K, Goetsch L, Byers B, Futcher B: Characterization of four B-type cyclin genes of the budding yeast Saccharomyces cerevisiae, Mol Biol Cell 1992, 3:805-818

55. Korolyev E, Steinberg-Neifach O, Eshel D: Mutations in the yeast kinesin-like Cin8p are alleviated by osmotic support, FEMS Microbiol Lett 2005, 244:379-383

56. Gheber L, Kuo SC, Hoyt MA: Motile properties of the kinesin-related Cin8p spindle motor extracted from Saccharomyces cerevisiae cells, J Biol Chem 1999, 274:9564-9572

57. Crasta K, Huang P, Morgan G, Winey M, Surana U: Cdk1 regulates centrosome separation by restraining proteolysis of microtubule-associated proteins, EMBO J 2006, 25:2551-2563

58. Steinberg-Neifach O, Eshel D: Simultaneous expression of both MAT loci in haploid cells suppresses mutations in yeast microtubule motor genes, Mol Gen Genet 2000, 264:300-305

59. Carlson M, Botstein D: Two differentially regulated mRNAs with different 5' ends encode secreted with intracellular forms of yeast invertase, Cell 1982, 28:145-154

60. Broach JR: The Yeast Plasmid, 2μ Circle. Edited by Cold Spring Harbor Laboratory Press, 1981, p. pp. 445-470

61. Rine J, Hansen W, Hardeman E, Davis RW: Targeted selection of recombinant clones through gene dosage effects, Proc Natl Acad Sci U S A 1983, 80:6750-6754

62. Steinberg-Neifach O, Eshel D: Heterozygosity in MAT locus affects stability and function of microtubules in yeast, Biol Cell 2002, 94:147-156

63. Kaiser CM, S., Mitchell, A.: Methods in Yeast Genetics: A Laboratory Course Manual. Edited by 1994, p.

64. Gietz RD, Schiestl RH, Willems AR, Woods RA: Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure, Yeast 1995, 11:355-360

65. Tiwari A, Bhat JP: Molecular characterization reveals that YMR278w encoded protein is environmental stress response homologue of Saccharomyces cerevisiae PGM2, Biochem Biophys Res Commun 2008, 366:340-345

66. Kobor MS, Archambault J, Lester W, Holstege FC, Gileadi O, Jansma DB, Jennings EG, Kouyoumdjian F, Davidson AR, Young RA, Greenblatt J: An unusual eukaryotic protein phosphatase required for transcription by RNA polymerase II and CTD dephosphorylation in S. cerevisiae, Mol Cell 1999, 4:55-62

67. Ausubel FM, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl: Current Protocols in Molecular Biology. Edited by Greene Publishing Associates/Wiley Interscience, New-York, 1987, p.

68. Solomon F CL, Kirkpatrick D, Praitis, V and Weinstein, B: Methods for Studying the Yeast Cytoskeleton. Edited by Oxford, Oxford University Press, 1992, p.

69. Thomas JH, Neff NF, Botstein D: Isolation and characterization of mutations in the beta-tubulin gene of Saccharomyces cerevisiae, Genetics 1985, 111:715-734

70. Machin NA, Lee JM, Barnes G: Microtubule stability in budding yeast: characterization and dosage suppression of a benomyl-dependent tubulin mutant, Mol Biol Cell 1995, 6:1241-1259

71. Adames NR, Cooper JA: Microtubule interactions with the cell cortex causing nuclear movements in Saccharomyces cerevisiae, J Cell Biol 2000, 149:863-874

72. Kull FJ, Sablin EP, Lau R, Fletterick RJ, Vale RD: Crystal structure of the kinesin motor domain reveals a structural similarity to myosin, Nature 1996, 380:550-555

73. Ghosh A, Shuman S, Lima CD: The structure of Fcp1, an essential RNA polymerase II CTD phosphatase, Mol Cell 2008, 32:478-490

74. Dahmus ME: Reversible phosphorylation of the C-terminal domain of RNA polymerase II, J Biol Chem 1996, 271:19009-19012

75. Kobor MS, Simon LD, Omichinski J, Zhong G, Archambault J, Greenblatt J: A motif shared by TFIIF and TFIIB mediates their interaction with the RNA polymerase II carboxy-terminal domain phosphatase Fcp1p in Saccharomyces cerevisiae, Mol Cell Biol 2000, 20:7438-7449

76. Dubois MF, Nguyen VT, Bellier S, Bensaude O: Inhibitors of transcription such as 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole and isoquinoline sulfonamide derivatives (H-8 and H-7) promote dephosphorylation of the carboxyl-terminal domain of RNA polymerase II largest subunit, J Biol Chem 1994, 269:13331-13336

77. Mandal SS, Cho H, Kim S, Cabane K, Reinberg D: FCP1, a phosphatase specific for the heptapeptide repeat of the largest subunit of RNA polymerase II, stimulates transcription elongation, Mol Cell Biol 2002, 22:7543-7552

78. Collet JF, Stroobant V, Pirard M, Delpierre G, Van Schaftingen E: A new class of phosphotransferases phosphorylated on an aspartate residue in an amino-terminal DXDX(T/V) motif, J Biol Chem 1998, 273:14107-14112

79. Ishikawa M, Janda M, Krol MA, Ahlquist P: In vivo DNA expression of functional brome mosaic virus RNA replicons in Saccharomyces cerevisiae, J Virol 1997, 71:7781-7790

80. Su YA, Lee MM, Hutter CM, Meltzer PS: Characterization of a highly conserved gene (OS4) amplified with CDK4 in human sarcomas, Oncogene 1997, 15:1289-1294

81. Bork P, Hofmann K, Bucher P, Neuwald AF, Altschul SF, Koonin EV: A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins, FASEB J 1997, 11:68-76

82. Bang W, Kim S, Ueda A, Vikram M, Yun D, Bressan RA, Hasegawa PM, Bahk J, Koiwa H: Arabidopsis carboxyl-terminal domain phosphatase-like isoforms share common catalytic and interaction domains but have distinct in planta functions, Plant Physiol 2006, 142:586-594

83. Kamenski T, Heilmeier S, Meinhart A, Cramer P: Structure and mechanism of RNA polymerase II CTD phosphatases, Mol Cell 2004, 15:399-407

84. Archambault J, Chambers RS, Kobor MS, Ho Y, Cartier M, Bolotin D, Andrews B, Kane CM, Greenblatt J: 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 1997, 94:14300-14305

85. Ohkuni K, Okuda A, Kikuchi A: Yeast Nap1-binding protein Nbp2p is required for mitotic growth at high temperatures and for cell wall integrity, Genetics 2003, 165:517-529

86. Krishnamurthy S, He X, Reyes-Reyes M, Moore C, Hampsey M: Ssu72 Is an RNA polymerase II CTD phosphatase, Mol Cell 2004, 14:387-394

87. Burke D, Gasdaska P, Hartwell L: Dominant effects of tubulin overexpression in Saccharomyces cerevisiae, Mol Cell Biol 1989, 9:1049-1059

88. Weinstein B, Solomon F: Phenotypic consequences of tubulin overproduction in Saccharomyces cerevisiae: differences between alpha-tubulin and beta-tubulin, Mol Cell Biol 1990, 10:5295-5304

89. Adams AE, Cooper JA, Drubin DG: Unexpected combinations of null mutations in genes encoding the actin cytoskeleton are lethal in yeast, Mol Biol Cell 1993, 4:459-468

90. Knop M, Pereira G, Geissler S, Grein K, Schiebel E: The spindle pole body component Spc97p interacts with the gamma-tubulin of Saccharomyces cerevisiae and functions in microtubule organization and spindle pole body duplication, EMBO J 1997, 16:1550-1564

91. Straight AF, Sedat JW, Murray AW: Time-lapse microscopy reveals unique roles for kinesins during anaphase in budding yeast, J Cell Biol 1998, 143:687-694

92. Hoyt MA, He L, Totis L, Saunders WS: Loss of function of Saccharomyces cerevisiae kinesin-related CIN8 and KIP1 is suppressed by KAR3 motor domain mutations, Genetics 1993, 135:35-44

93. Li YY, Yeh E, Hays T, Bloom K: Disruption of mitotic spindle orientation in a yeast dynein mutant, Proc Natl Acad Sci U S A 1993, 90:10096-10100

94. Roof DM, Meluh PB, Rose MD: Kinesin-related proteins required for assembly of the mitotic spindle, J Cell Biol 1992, 118:95-108

95. Kumar A, Seringhaus M, Biery MC, Sarnovsky RJ, Umansky L, Piccirillo S, Heidtman M, Cheung KH, Dobry CJ, Gerstein MB, Craig NL, Snyder M: Large-scale mutagenesis of the yeast genome using a Tn7-derived multipurpose transposon, Genome Res 2004, 14:1975-1986

96. Gonzalez-Ballester D, de Montaigu A, Galvan A, Fernandez E: Restriction enzyme site-directed amplification PCR: a tool to identify regions flanking a marker DNA, Anal Biochem 2005, 340:330-335

97. Venema J, Tollervey D: Ribosome synthesis in Saccharomyces cerevisiae, Annu Rev Genet 1999, 33:261-311

98. Johnston M, Hillier L, Riles L, Albermann K, Andre B, Ansorge W, Benes V, Bruckner M, Delius H, Dubois E, Dusterhoft A, Entian KD, Floeth M, Goffeau A, Hebling U, Heumann K, Heuss-Neitzel D, Hilbert H, Hilger F, Kleine K, Kotter P, Louis EJ, Messenguy F, Mewes HW, Hoheisel JD, et al.: The nucleotide sequence of Saccharomyces cerevisiae chromosome XII, Nature 1997, 387:87-90

99. Witter DJ, Poulter CD: Yeast geranylgeranyltransferase type-II: steady state kinetic studies of the recombinant enzyme, Biochemistry 1996, 35:10454-10463

100. Miaczynska M, Lorenzetti S, Bialek U, Benito-Moreno RM, Schweyen RJ, Ragnini A: The yeast Rab escort protein binds intracellular membranes in vivo and in vitro, J Biol Chem 1997, 272:16972-16977

101. Stenmark H: Rab GTPases as coordinators of vesicle traffic, Nat Rev Mol Cell Biol 2009, 10:513-525

102. Jiang Y, Ferro-Novick S: Identification of yeast component A: reconstitution of the geranylgeranyltransferase that modifies Ypt1p and Sec4p, Proc Natl Acad Sci U S A 1994, 91:4377-4381

103. Schmitt HD, Wagner P, Pfaff E, Gallwitz D: The ras-related YPT1 gene product in yeast: a GTP-binding protein that might be involved in microtubule organization, Cell 1986, 47:401-412

104. Bialek-Wyrzykowska U, Bauer BE, Wagner W, Kohlwein SD, Schweyen RJ, Ragnini A: Low levels of Ypt protein prenylation cause vesicle polarization defects and thermosensitive growth that can be suppressed by genes involved in cell wall maintenance, Mol Microbiol 2000, 35:1295-1311

105. Horak CE, Luscombe NM, Qian J, Bertone P, Piccirrillo S, Gerstein M, Snyder M: Complex transcriptional circuitry at the G1/S transition in Saccharomyces cerevisiae, Genes Dev 2002, 16:3017-3033

106. Costanzo M, Schub O, Andrews B: G1 transcription factors are differentially regulated in Saccharomyces cerevisiae by the Swi6-binding protein Stb1, Mol Cell Biol 2003, 23:5064-5077

107. Cooper K: Rb, whi it's not just for metazoans anymore, Oncogene 2006, 25:5228-5232

108. de Bruin RA, McDonald WH, Kalashnikova TI, Yates J, 3rd, Wittenberg C: Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5, Cell 2004, 117:887-898

109. Costanzo M, Nishikawa JL, Tang X, Millman JS, Schub O, Breitkreuz K, Dewar D, Rupes I, Andrews B, Tyers M: CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast, Cell 2004, 117:899-913

110. Chee MK, Haase SB: B-cyclin/CDKs regulate mitotic spindle assembly by phosphorylating kinesins-5 in budding yeast, PLoS Genet 6:e1000935

111. Wong J, Nakajima Y, Westermann S, Shang C, Kang JS, Goodner C, Houshmand P, Fields S, Chan CS, Drubin D, Barnes G, Hazbun T: A protein interaction map of the mitotic spindle, Mol Biol Cell 2007, 18:3800-3809

112. Geymonat M, Spanos A, Wells GP, Smerdon SJ, Sedgwick SG: Clb6/Cdc28 and Cdc14 regulate phosphorylation status and cellular localization of Swi6, Mol Cell Biol 2004, 24:2277-2285

113. Amon A, Tyers M, Futcher B, Nasmyth K: Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins, Cell 1993, 74:993-1007

114. Bailly E, Cabantous S, Sondaz D, Bernadac A, Simon MN: Differential cellular localization among mitotic cyclins from Saccharomyces cerevisiae: a new role for the axial budding protein Bud3 in targeting Clb2 to the mother-bud neck, J Cell Sci 2003, 116:4119-4130

115. Verma R, Annan RS, Huddleston MJ, Carr SA, Reynard G, Deshaies RJ: Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase, Science 1997, 278:455-460

116. Schwob E, Nasmyth K: CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae, Genes Dev 1993, 7:1160-1175

117. Donaldson AD, Raghuraman MK, Friedman KL, Cross FR, Brewer BJ, Fangman WL: CLB5-dependent activation of late replication origins in S. cerevisiae, Mol Cell 1998, 2:173-182

118. Richardson H, Lew DJ, Henze M, Sugimoto K, Reed SI: Cyclin-B homologs in Saccharomyces cerevisiae function in S phase and in G2, Genes Dev 1992, 6:2021-2034

119. Dynlacht BD, Moberg K, Lees JA, Harlow E, Zhu L: Specific regulation of E2F family members by cyclin-dependent kinases, Mol Cell Biol 1997, 17:3867-3875

120. Nachmias A, Barash I: Decreased permeability as a mechanism of resistance to methyl benzimadazol-2-yl carbamate (MBC) in Sporobolomyces roseus, J Gen Microbiol 1976, 94:167-172

121. Sanchez-Torres PaT, JJ: Molecular Insight into Fungicide Resistance in Sensitive and Resistant Penicillium digitatum Strains Infecting Citrus, Postharvest Biology and Technology 2011, 59:159-165

122. MacDonald LM, Armson A, Thompson AR, Reynoldson JA: Characterisation of benzimidazole binding with recombinant tubulin from Giardia duodenalis, Encephalitozoon intestinalis, and Cryptosporidium parvum, Mol Biochem Parasitol 2004, 138:89-96

123. Crasta K, Lim HH, Giddings TH, Jr., Winey M, Surana U: Inactivation of Cdh1 by synergistic action of Cdk1 and polo kinase is necessary for proper assembly of the mitotic spindle, Nat Cell Biol 2008, 10:665-675

124. Hildebrandt ER, Hoyt MA: Cell cycle-dependent degradation of the Saccharomyces cerevisiae spindle motor Cin8p requires APC(Cdh1) and a bipartite destruction sequence, Mol Biol Cell 2001, 12:3402-3416

125. Eluere R, Offner N, Varlet I, Motteux O, Signon L, Picard A, Bailly E, Simon MN: Compartmentalization of the functions and regulation of the mitotic cyclin Clb2 in S. cerevisiae, J Cell Sci 2007, 120:702-711

126. Douglas LM, Alvarez FJ, McCreary C, Konopka JB: Septin function in yeast model systems and pathogenic fungi, Eukaryot Cell 2005, 4:1503-1512

127. Booher RN, Deshaies RJ, Kirschner MW: Properties of Saccharomyces cerevisiae wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins, EMBO J 1993, 12:3417-3426

128. Kusch J, Meyer A, Snyder MP, Barral Y: Microtubule capture by the cleavage apparatus is required for proper spindle positioning in yeast, Genes Dev 2002, 16:1627-1639

129. Bouquin N, Barral Y, Courbeyrette R, Blondel M, Snyder M, Mann C: Regulation of cytokinesis by the Elm1 protein kinase in Saccharomyces cerevisiae, J Cell Sci 2000, 113 (Pt 8):1435-1445

130. McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JP, Hirschhorn JN: Genome-wide association studies for complex traits: consensus, uncertainty and challenges, Nat Rev Genet 2008, 9:356-369

131. Manderson EN, Malleshaiah M, Michnick SW: A novel genetic screen implicates Elm1 in the inactivation of the yeast transcription factor SBF, PLoS One 2008, 3:e1500

132. Edgington NP, Blacketer MJ, Bierwagen TA, Myers AM: Control of Saccharomyces cerevisiae filamentous growth by cyclin-dependent kinase Cdc28, Mol Cell Biol 1999, 19:1369-1380

133. Sutherland CM, Hawley SA, McCartney RR, Leech A, Stark MJ, Schmidt MC, Hardie DG: Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex, Curr Biol 2003, 13:1299-1305

134. Szkotnicki L, Crutchley JM, Zyla TR, Bardes ES, Lew DJ: The checkpoint kinase Hsl1p is activated by Elm1p-dependent phosphorylation, Mol Biol Cell 2008, 19:4675-4686

135. Caydasi AK, Kurtulmus B, Orrico MI, Hofmann A, Ibrahim B, Pereira G: Elm1 kinase activates the spindle position checkpoint kinase Kin4, J Cell Biol 190:975-989

136. Lew DJ, Burke DJ: The spindle assembly and spindle position checkpoints, Annu Rev Genet 2003, 37:251-282

137. Fraschini R, Venturetti M, Chiroli E, Piatti S: The spindle position checkpoint: how to deal with spindle misalignment during asymmetric cell division in budding yeast, Biochem Soc Trans 2008, 36:416-420

138. Siller KH, Doe CQ: Spindle orientation during asymmetric cell division, Nat Cell Biol 2009, 11:365-374

139. Grava S, Schaerer F, Faty M, Philippsen P, Barral Y: Asymmetric recruitment of dynein to spindle poles and microtubules promotes proper spindle orientation in yeast, Dev Cell 2006, 10:425-439

140. Barral Y: Mitosis: FEAR pulls them apart, Dev Cell 2004, 6:608-610

141. Maekawa H, Usui T, Knop M, Schiebel E: Yeast Cdk1 translocates to the plus end of cytoplasmic microtubules to regulate bud cortex interactions, EMBO J 2003, 22:438-449

142. Liakopoulos D, Kusch J, Grava S, Vogel J, Barral Y: Asymmetric loading of Kar9 onto spindle poles and microtubules ensures proper spindle alignment, Cell 2003, 112:561-574

143. Koch C, Schleiffer A, Ammerer G, Nasmyth K: Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at start, whereas Clb/Cdc28 kinases displace it from the promoter in G2, Genes Dev 1996, 10:129-141

144. Siegmund RF, Nasmyth KA: The Saccharomyces cerevisiae Start-specific transcription factor Swi4 interacts through the ankyrin repeats with the mitotic Clb2/Cdc28 kinase and through its conserved carboxy terminus with Swi6, Mol Cell Biol 1996, 16:2647-2655

145. Sidorova JM, Mikesell GE, Breeden LL: Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization, Mol Biol Cell 1995, 6:1641-1658

146. Gordon O, Taxis C, Keller PJ, Benjak A, Stelzer EH, Simchen G, Knop M: Nud1p, the yeast homolog of Centriolin, regulates spindle pole body inheritance in meiosis, EMBO J 2006, 25:3856-3868

147. Geymonat M, Spanos A, Walker PA, Johnston LH, Sedgwick SG: In vitro regulation of budding yeast Bfa1/Bub2 GAP activity by Cdc5, J Biol Chem 2003, 278:14591-14594

148. Buratowski S: The CTD code, Nat Struct Biol 2003, 10:679-680

149. Fabrega C, Shen V, Shuman S, Lima CD: Structure of an mRNA capping enzyme bound to the phosphorylated carboxy-terminal domain of RNA polymerase II, Mol Cell 2003, 11:1549-1561

150. Jeong SJ, Kim HJ, Yang YJ, Seol JH, Jung BY, Han JW, Lee HW, Cho EJ: Role of RNA polymerase II carboxy terminal domain phosphorylation in DNA damage response, J Microbiol 2005, 43:516-522

151. Kimura M, Suzuki H, Ishihama A: Formation of a carboxy-terminal domain phosphatase (Fcp1)/TFIIF/RNA polymerase II (pol II) complex in Schizosaccharomyces pombe involves direct interaction between Fcp1 and the Rpb4 subunit of pol II, Mol Cell Biol 2002, 22:1577-1588

152. Palancade B, Dubois MF, Dahmus ME, Bensaude O: Transcription-independent RNA polymerase II dephosphorylation by the FCP1 carboxy-terminal domain phosphatase in Xenopus laevis early embryos, Mol Cell Biol 2001, 21:6359-6368

153. Gallicano GI: Composition, regulation, and function of the cytoskeleton in mammalian eggs and embryos, Front Biosci 2001, 6:D1089-1108

154. Adams RR, Carmena M, Earnshaw WC: Chromosomal passengers and the (aurora) ABCs of mitosis, Trends Cell Biol 2001, 11:49-54

155. Canman JC, Cameron LA, Maddox PS, Straight A, Tirnauer JS, Mitchison TJ, Fang G, Kapoor TM, Salmon ED: Determining the position of the cell division plane, Nature 2003, 424:1074-1078

156. Bouck DC, Bloom KS: The kinetochore protein Ndc10p is required for spindle stability and cytokinesis in yeast, Proc Natl Acad Sci U S A 2005, 102:5408-5413

157. Gachet Y, Tournier S, Millar JB, Hyams JS: Mechanism controlling perpendicular alignment of the spindle to the axis of cell division in fission yeast, EMBO J 2004, 23:1289-1300

158. Franco A, Meadows JC, Millar JB: The Dam1/DASH complex is required for the retrieval of unclustered kinetochores in fission yeast, J Cell Sci 2007, 120:3345-3351

159. Tournier S, Gachet Y, Buck V, Hyams JS, Millar JB: Disruption of astral microtubule contact with the cell cortex activates a Bub1, Bub3, and Mad3-dependent checkpoint in fission yeast, Mol Biol Cell 2004, 15:3345-3356

160. King MC, Drivas TG, Blobel G: A network of nuclear envelope membrane proteins linking centromeres to microtubules, Cell 2008, 134:427-438

161. Huisman SM, Smeets MF, Segal M: Phosphorylation of Spc110p by Cdc28p-Clb5p kinase contributes to correct spindle morphogenesis in S. cerevisiae, J Cell Sci 2007, 120:435-446

162. Yamashita A, Sato M, Fujita A, Yamamoto M, Toda T: The roles of fission yeast ase1 in mitotic cell division, meiotic nuclear oscillation, and cytokinesis checkpoint signaling, Mol Biol Cell 2005, 16:1378-1395

163. De Souza CP, Osmani SA: Mitosis, not just open or closed, Eukaryot Cell 2007, 6:1521-1527

164. Zeng X, Kahana JA, Silver PA, Morphew MK, McIntosh JR, Fitch IT, Carbon J, Saunders WS: Slk19p is a centromere protein that functions to stabilize mitotic spindles, J Cell Biol 1999, 146:415-425

165. de Gramont A, Barbour L, Ross KE, Cohen-Fix O: The spindle midzone ıe number and orientation of astral microtubules in Saccharomyces cerevisiae, Cell Cycle 2007, 6:1231-1241

166. Perez-Perez JM, Candela H, Micol JL: Understanding synergy in genetic interactions, Trends Genet 2009, 25:368-376

167. Rutherford SL: From genotype to phenotype: buffering mechanisms and the storage of genetic information, Bioessays 2000, 22:1095-1105