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# Budding Yeast *SKP1* Encodes an Evolutionarily Conserved Kinetochores Protein Required for Cell Cycle Progression

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## Summary

The budding yeast *SKP1* gene, identified as a dosage suppressor of a known kinetochores protein mutant, encodes an intrinsic 22.3 kDa subunit of CBF3, a multi-protein complex that binds centromere DNA *in vitro*. Temperature-sensitive mutations in *SKP1* define two distinct phenotypic classes. *skp1-4* mutants arrest predominantly as large budded cells with a G2 DNA content and short mitotic spindle, consistent with a role in kinetochores function. *skp1-3* mutants, however, arrest predominantly as multiply budded cells with a G1 DNA content, suggesting an additional role during the G1/S phase. Identification of Skp1p homologs from *C. elegans*, *A. thaliana*, and *H. sapiens* indicates that *SKP1* is evolutionarily highly conserved. Skp1p therefore represents an intrinsic kinetochores protein conserved throughout eukaryotic evolution and may be directly involved in linking kinetochores function with the cell cycle-regulatory machinery.

## Introduction

Centromeres (also termed kinetochores) are of fundamental importance to chromosomal inheritance in all eukaryotes (reviewed by Pluta et al., 1995). These functionally differentiated structures on eukaryotic chromosomes consist of specific centromere DNA sequences and associated proteins that facilitate binding to the spindle microtubules. Kinetochores mediate chromosome movement to ensure the ordered segregation of sister chromatids into daughter cells during mitosis and meiosis. Kinetochores activity must be regulated to coordinate chromosome segregation with other events in the cell cycle and to ensure that bipolar attachment of all chromosomes is achieved before anaphase begins. The structural complexity of centromeres varies widely among higher and lower eukaryotes, as exemplified by the centromeres of animal cells (which span several thousand kilobases of DNA) and budding yeast (which span <0.2 kb of DNA). Despite these apparent organizational differences, centromeres in higher and lower eukaryotes are likely to share evolutionarily conserved components.

The structurally compact centromere of the budding yeast, *Saccharomyces cerevisiae*, has been analyzed extensively using molecular genetic techniques (reviewed by Hegemann and Fleig, 1993). The centromere DNA sequence encompasses a 125 bp region that includes three conserved elements, termed CDEI, CDEII, and CDEIII (Fitzgerald-Hayes et al., 1982; Hieter et al., 1985). CDEIII (25 bp) is absolutely essential, as evidenced by single point mutations that destroy centromere activity (Hegemann et al., 1988). Lechner and Carbon (1991) described the purification of a 240 kDa multicomponent protein complex, termed CBF3, that

specifically binds CDEIII sequences *in vitro*. The CBF3 complex has three major components that are 110, 64, and 58 kDa in size. The genes encoding p110 (*NDC10/CBF2/CTF14*) (Goh and Kilmartin, 1993; Jiang et al., 1993; Doheny et al., 1993), p64 (*CEP3/CBF3b*) (Strunnikov et al., 1995; Lechner, 1994), and p58 (*CTF13*) (Doheny et al., 1993) have been cloned and characterized. All three proteins are essential for viability, and mutations in any one of them cause a chromosome missegregation phenotype, consistent with a role in kinetochores function. At nonpermissive temperature, the *ndc10-1* mutation causes asymmetric segregation of chromosomes in which all chromosomes segregate to one pole following nuclear division. Temperature-sensitive mutations in *ctf13* or *cep3/cbf3c* exhibit G2/M accumulation at nonpermissive temperature with preanaphase short mitotic spindles. Cep3p contains a predicted zinc finger and may therefore represent a DNA-binding component. Ndc10p contains a GTP-binding motif. None of the three CBF3 proteins described to date exhibits extensive homology to any known proteins in the databases.

The coordination of centromere function with cell cycle progression is likely to require regulation of kinetochores protein activity and a signal transduction system that monitors completion of chromosome attachment and relays the status of events to the cell cycle machinery. A direct role for the kinetochores in a checkpoint that monitors completion of metaphase is suggested by studies in both animal and yeast cells. Microinjection of anti-centromere protein antibodies into cultured human cells induces mitotic delay (Bernat et al., 1990). In cultured mammalian cells, the onset of anaphase following nuclear envelope breakdown is delayed even when only a single kinetochores is not attached to microtubules (Rieder et al., 1994). Laser ablation of the unattached kinetochores destroys the delay in anaphase onset, suggesting an inhibitory effect of the unattached kinetochores in cell cycle progression. In yeast cells, a preanaphase delay is induced by the presence of a single mutant centromere DNA (Spencer and Hieter, 1992) or by hypomorphic mutations in kinetochores proteins (Doheny et al., 1993). Recently, three groups have presented evidence that the preanaphase delay induced by abnormal kinetochores function requires genes that are involved in a mitotic checkpoint-monitoring spindle assembly (Wang and Burke, 1995; Wells and Murray, 1996; Pangilinan and Spencer, 1996). Very little is currently known about the molecular components or mechanism that signals defects in kinetochores structure to cell cycle regulators.

Kinetochores execute a variety of activities, including binding of kinetochores protein complexes to centromere DNA, binding of *CEN* DNA-kinetochores protein complexes to microtubules, movement of chromosomes along the microtubule fibers, and responses to and involvement in cell cycle regulation. Given this complexity, additional structural and regulatory components must be identified to gain a complete understanding of kinetochores function. To accomplish this, we have employed a variety of genetic screens in *S. cerevisiae* using *ctf13-30*, a temperature-sensitive mutation in the

gene encoding p58 of CBF3, as a starting point. In this paper, we describe the identification of the *SKP1* gene as a dosage suppressor of a *ctf13-30* mutant. Genetic and biochemical analysis indicates that the 22.3 kDa Skp1 protein is a fourth subunit of CBF3 that was previously unrecognized. Unlike the other CBF3 components, Skp1p is a highly conserved protein found in multicellular eukaryotes. Furthermore, Skp1p has recently been shown to be a component of a cyclin A-cyclin-dependent kinase 2 (CDK2) complex purified from transformed human fibroblasts (Zhang et al., 1995) and to interact directly with Cdc4p and cyclin F through a novel structural motif (Bai et al., 1996 [this issue of *Cell*]). We conclude that Skp1p is involved in both structural and regulatory functions and may provide a direct link between the kinetochore and the cell cycle machinery.

**Results**

**High Copy Suppression of a Known Kinetochore Component**

To identify further additional components of the *S. cerevisiae* kinetochore, we performed a screen for dosage suppressors of the temperature sensitivity caused by the *ctf13-30* mutation. A 2 $\mu$  genomic library was transformed into a *ctf13-30* mutant, and temperature-resistant transformants were selected (see Experimental Procedures). We obtained 12 plasmid clones that fell into two classes of overlapping genomic segments: 7 of 12 corresponded to the *CTF13* gene; 5 of 12 contained a new locus. We named the locus *SKP1*, for suppressor of kinetochore protein 1. In addition to dosage suppression of the temperature sensitivity for growth, increased dosage of *SKP1* suppressed the chromosome missegregation phenotype caused by the *ctf13-30* mutation at semi-permissive temperature, even when *SKP1* was introduced at a modestly increased copy number on a centromere vector (data not shown). Insertion of a DNA fragment containing a prototrophic marker into a unique NsiI site disrupted the ability of the genomic plasmid clone to suppress the temperature-sensitive and chromosome missegregation phenotypes of a *ctf13-30* mutant. When the gene disruption was integrated into the genome, dissection of a heterozygous diploid resulted in viability segregating 2+ : 2- (see Experimental Procedures). The genomic clone was localized to the right arm of chromosome IV using physical mapping methods, indicating that *SKP1* is a previously unidentified essential gene in *S. cerevisiae*.

The nucleotide sequence of the *SKP1* genomic DNA contains a 582 bp open reading frame (ORF) that encodes a protein of 194 amino acids with a predicted molecular mass of 22.3 kDa (Figure 1A). The restriction maps of genomic *SKP1* and derivative clones are shown in Figure 1.

***SKP1* Encodes a Fourth Subunit of CBF3**

Lechner and Carbon (1991) described a multiprotein complex (CBF3) present in extracts of *S. cerevisiae* cells that contains three major protein species 110, 64, and 58 kDa in size. CBF3 binds in vitro to a 100 bp DNA probe that spans CDEIII but lacks CDEI and CDEII (Doheny et al., 1993; see Experimental Procedures). Because of the

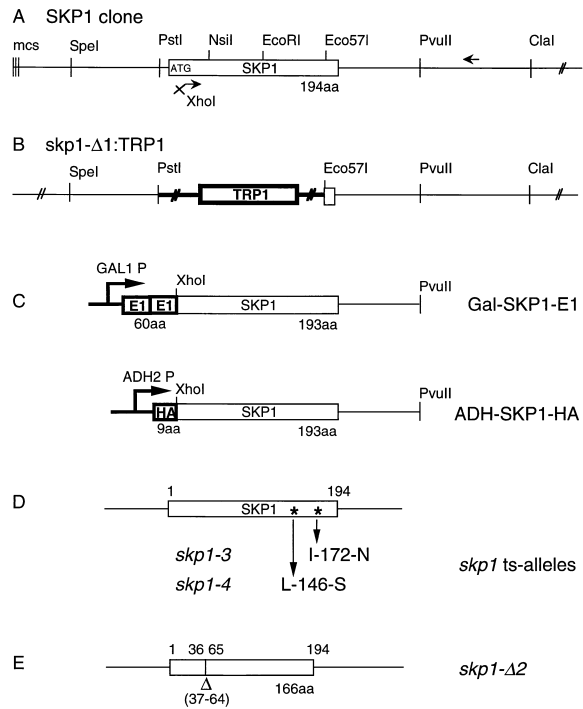


Figure 1. *SKP1* Genomic DNA, Mutant Alleles, and Epitope Tag Fusion Proteins

(A) Restriction map of the cloned *SKP1* gene and flanking regions. The vector multiple cloning site (mcs) represents the original left end of the library clone where a genomic *Sau3A* site was ligated to a *Bam*HI site in the vector.  
 (B) The *skp1-Δ1* allele was constructed by replacement of the 0.73 kb *Pst*I-*Eco*57I segment with a 1.8 kb *TRP1* gene containing fragment (shown in bold).  
 (C) Schematic structures of the E1 and HA amino-terminal epitope-tagged Skp1p fusion constructs. The E1-Skp1p fusion is under transcriptional control of the *GAL1* promoter; the HA-Skp1p fusion is under the transcriptional control of the *ADH2* promoter.  
 (D) Schematic of the *skp1* temperature-sensitive alleles used in phenotypic studies.  
 (E) Schematic of *skp1-Δ2*. The 28 amino acid in-frame deletion corresponds to amino acid residues 37-64.

small size of Skp1p, it was possible that it represented a previously unrecognized subunit of CBF3. To determine whether this was the case, we made two epitope-tagged constructs of Skp1p (see Experimental Procedures). In the first, two tandem copies of the E1 epitope (Pluta et al., 1992), derived from the carboxy-terminal 25 amino acids of avian coronavirus glycoprotein (Machamer and Rose, 1987), were placed in-frame at the amino terminus of the *SKP1* ORF under the transcriptional control of the *GAL1* promoter (Figure 1C). In the second construct, a 9 amino acid epitope derived from the HA1 protein of influenza virus (Field et al., 1988) was placed in-frame at the amino terminus of the *SKP1* ORF under the control of the *ADH2* promoter (Figure 1C). Both epitope-tagged Skp1p derivatives were able to rescue viability in a *skp1-Δ1::TRP1* deletion strain (Figure 1B).

Whole-cell extracts were prepared from a *skp1-Δ1* deletion strain carrying either epitope-tagged or untagged copies of Skp1p. As controls, extracts were also prepared from a *ctf13-Δ1* deletion strain carrying epitope-tagged Ctf13p. DNA-protein complexes were allowed to form with <sup>32</sup>P-labeled CDEIII sequence, and

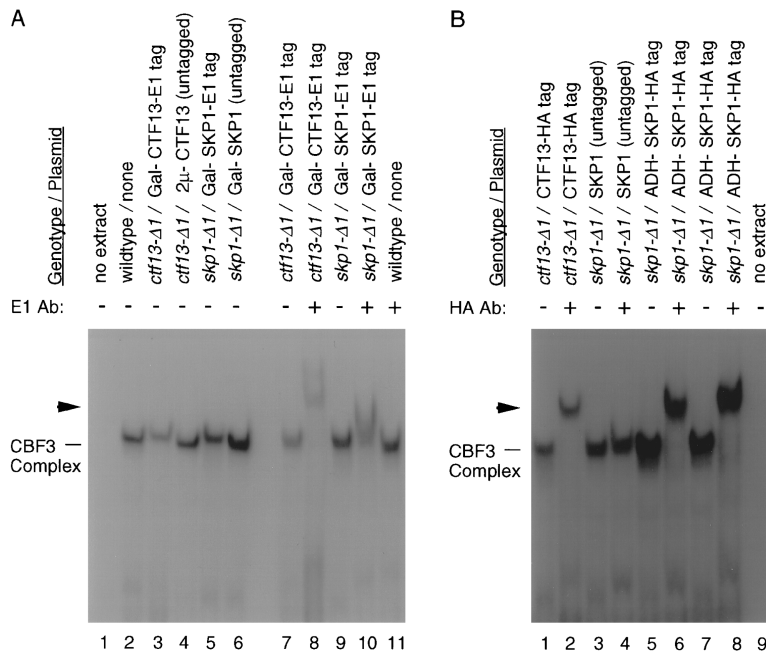


Figure 2. Mobility Shift Analysis of Skp1p in CBF3-CEN DNA Complexes

DNA-protein complexes formed with <sup>32</sup>P-labeled CDEIII probe and whole-cell extracts were analyzed on a non-denaturing polyacrylamide gel (Doheny et al., 1993). Antibodies were added to preformed complexes, and samples were incubated for 20 min at room temperature before gel analysis. Unbound probe was run off the bottom of the gel.

(A) Lanes 1, 2, and 11 are controls. Lanes 3-6, extracts from cells containing E1-tagged or untagged Ctf13p or Skp1p proteins demonstrating an intrinsic mobility shift due to the 60 amino acid epitope fusion in each case. Lanes 7-10, extracts from cells containing E1-tagged Ctf13p or Skp1p subsequently incubated with or without antibodies directed against the E1 epitope as indicated. Lanes 7 and 9 are controls for lanes 8 and 10.

(B) Lanes 1-6, extracts containing either HA-tagged or untagged Ctf13p or Skp1p subsequently incubated with or without an antibody directed against the HA epitope as indicated. Lanes 7 and 8 are from an independent transformant (repeat of lanes 5 and 6). Lane 9 is a control.

complexes were resolved on a non-denaturing gel (Figure 2). In the absence of anti-E1 antibody, extracts containing the E1 epitope-tagged fusions of Ctf13p and Skp1p show a slight (but clearly observable and reproducible) intrinsic mobility shift up when compared with wild-type and untagged extracts (Figure 2A, lanes 2-6), suggesting that the E1-Skp1p fusion protein is localized to the complex. The magnitude of the intrinsic shift is identical for E1-tagged Ctf13p and E1-tagged Skp1p, indicating equal stoichiometry of these two proteins in the CBF3 complex. When E1 antibody is added to complexes formed using extracts from tagged and untagged Ctf13p and Skp1p strains, a supershift is observed using the Ctf13p-tagged extract (Figure 2A, lane 8) and the E1-tagged Skp1p extract, although the supershift with E1-tagged Skp1p was less pronounced (Figure 2A, lane 10). These results suggested that Skp1p is in the CBF3 complex, as evidenced by both the intrinsic and antibody-induced mobility shift; however, epitope accessibility or antibody binding stability could have compromised a supershift equivalent to that seen with epitope-tagged Ctf13p.

The addition of anti-HA antibody to complexes formed using extracts prepared from untagged and HA-tagged fusions of Ctf13p and Skp1p showed conclusively that Skp1p is present in the CBF3-CEN DNA complex. Two independent extracts containing HA-tagged Skp1p produced a quantitative supershift equivalent to that seen for the HA-tagged Ctf13p (Figure 2B, lanes 2, 6, and 8). We conclude from these data that Skp1p is a fourth subunit of the *S. cerevisiae* CBF3 complex.

**Generation and Analysis of *skp1* Temperature-Sensitive Alleles**

To facilitate subsequent genetic and phenotypic analysis of mutants defective for SKP1 function, we generated four temperature-sensitive alleles of SKP1 using a method for random mutagenesis of yeast genes described by Muhrad et al. (1992) that involves mutagenic

polymerase chain reaction (PCR) in vitro and gap repair in vivo (see Experimental Procedures). The four alleles were named *skp1-1*, *skp1-2*, *skp1-3*, and *skp1-4*. The nucleotide sequence of each allele was determined (see Experimental Procedures), and the mutations encoding changes at the protein level were found to be primarily in the carboxyl terminus of the protein (see Table 1).

Chromosome missegregation was monitored visually in each *skp1* temperature-sensitive strain at semi-permissive temperature using a colony color assay that measures the stability of a marker chromosome fragment (Hieter et al., 1985). By this criterion, the strains fell into two classes. Strains expressing the *skp1-1*, *skp1-2*, or *skp1-3* alleles exhibited wild-type rates of chromosome missegregation. The strain expressing *skp1-4*, however, exhibited a dramatically increased rate of chromosome missegregation comparable with that observed for the *ctf13-30* mutant. We therefore decided to analyze further strains carrying one mutation in each class (*skp1-3* and *skp1-4*). Our approach was to integrate each mutant allele as a single copy into the genome to avoid potential plasmid copy number effects. Both the *skp1-3* and *skp1-4* alleles contain single missense mutations (Ile-172 to Asn and Leu-146 to Ser, respectively; Figure 1D). Each mutant allele was integrated at the *LEU2* locus in strains containing the *skp1-Δ1* deletion mutation (see Experimental Procedures).

The *skp1-3* and *skp1-4* mutations were found to cause several allele-specific phenotypes. First, as observed with the plasmid-borne alleles, *skp1-3* does not cause increased chromosome missegregation, while *skp1-4* causes a dramatic increase in chromosome missegregation (Figure 3, *skp1* mutants transformed with vector only). Chromosome transmission fidelity was quantitated in a *skp1-4/skp1-4* homozygous diploid using colony color half sector analysis (Koshland and Hieter, 1987). The rates of chromosome loss (1:0 segregation) and chromosome nondisjunction (2:0 segregation) in the *skp1-4* mutant were 3.9% and 1.7% per cell division,

Table 1. Mutations in *skp1* Temperature-Sensitive Alleles

Allele	Number of Mutations	Codon	Sequence Change	
			DNA	Protein
<i>skp1-1</i>	1	129	GAG to GGG	Glu to Gly
<i>skp1-2</i>	2	130	ATG to GTG	Met to Val
		176	TTC to TCC	Phe to Ser
<i>skp1-3</i>	1	172	ATC to AAC	Ile to Asn
<i>skp1-4</i>	3	146	TTG to TCG	Leu to Ser
		162	TCT to TCC	No change
		4	TCT to TCC	No change

respectively, which represent greater than 100-fold increases relative to wild type. Second, because *SKP1* was identified as a high copy suppressor of *ctf13-30*, it was of interest to test the reciprocal relationship, i.e., whether Ctf13p overexpression could suppress the *skp1* temperature-sensitive alleles. Overexpression of Ctf13p rescues the temperature sensitivity of *skp1-4*, but not that of *skp1-3* (data not shown). In addition, at semi-permissive temperature, the chromosome missegregation phenotype caused by the *skp1-4* mutation is suppressed by increased dosage of Ctf13p, even when present at one or a few extra copies on a centromere vector (Figure 3). This suppression is kinetochore protein gene specific, since increased dosage of Ndc10p has no effect, suggesting a direct interaction between the Skp1 and Ctf13 proteins. Third, following a 3 hr shift to nonpermissive temperature, *skp1-3* causes haploid cells to arrest with a G1 DNA content, while *skp1-4* causes haploid cells to arrest with a G2 DNA content (data not shown). These terminal phenotypes were more fully characterized in diploids (see below). Fourth, *skp1-3* and *skp1-4* mutations exhibit interallelic complementation. Heterozygous and homozygous diploids were created from meiotic segregants of each of the integrated temperature-sensitive alleles. At nonpermissive temperature (37°C), the *skp1-3/skp1-4* heteroallelic

diploid forms colonies, whereas either homozygous mutant diploid does not. Furthermore, at permissive temperature (25°C), the *skp1-3* allele complements the chromosome segregation defect of the *skp1-4* allele in the heteroallelic diploid.

Taken together these data strongly suggest two independent functions for the Skp1 protein: a G2/M function required for kinetochore activity and chromosome segregation and a G1/S function required for cell cycle progression earlier in the cell cycle.

**Cytological Analysis of *skp1* Mutants**

Cytological analysis of *skp1* mutants, and accompanying flow cytometric analysis of DNA content per cell, was performed in *skp1* homozygous and heteroallelic diploid mutants at permissive and nonpermissive temperatures (Figure 4). As compared with wild type, *skp1-3/skp1-3* mutants exhibit typical G1 and G2 profiles of DNA content per cell in logarithmically growing cultures at permissive temperature and arrest with a single peak of G1 content DNA after shift to nonpermissive temperature for 3 hr (Figure 4A). In contrast, *skp1-4/skp1-4* mutants exhibit a modest accumulation of cells with a G2 DNA content during log phase growth and arrest with a single peak of G2 content DNA after shift to the nonpermissive temperature. As expected from the intragenic

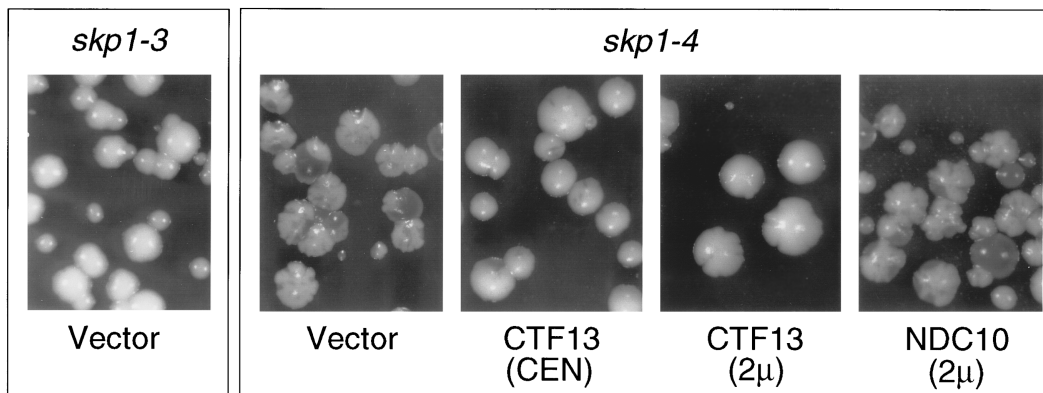


Figure 3. Chromosome Segregation Phenotypes of *skp1-3* and *skp1-4* Mutants

Yeast strains carrying integrated *skp1* temperature-sensitive alleles were tested for chromosome missegregation phenotypes using the colony color sectoring assay (Koshland and Hieter, 1987). Loss of the nonessential marker chromosome gives a red sector in a white colony. *skp1-3* mutants exhibit a wild-type phenotype (very rarely sectorial colonies) when transformed with vector only. *skp1-4* mutants exhibit dramatically increased rates of chromosome loss (highly sectorial colonies) when transformed with vector only. Introduction of the *CTF13* gene on a low copy (*CEN*) vector or high copy (2 $\mu$ ) vector suppresses the chromosome missegregation phenotype of the *skp1-4* mutant. Introduction of the *NDC10* gene on a 2 $\mu$  vector has no effect.

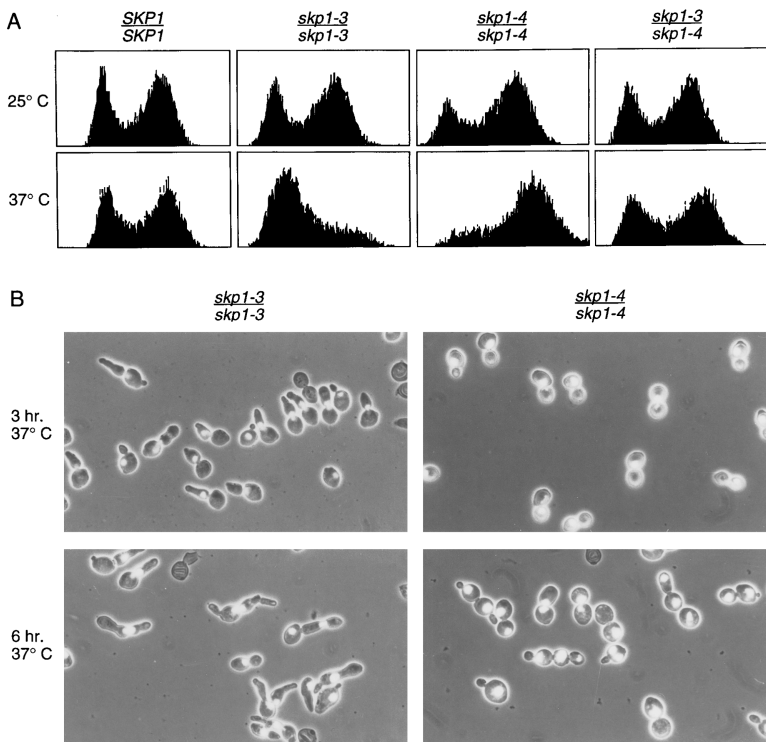


Figure 4. Phenotypic Analysis of *skp1* Temperature-Sensitive Mutants

(A) Shown are G1 arrest (at nonpermissive temperature) of *skp1-3* cells and G2/M accumulation (at permissive temperature) and G2/M arrest (at nonpermissive temperature) of *skp1-4* cells, as analyzed by flow cytometry (Gerring et al., 1990). The number of cells is depicted on the vertical axis, with fluorescent intensity of emitted light (proportional to DNA content) on the horizontal axis. Logarithmically growing cultures at 25°C were split and incubated at either 25°C or 37°C for 3 hr prior to analysis.

(B) Terminal arrest morphologies of *skp1-3* or *skp1-4* cells after 3 or 6 hr at 37°C. Cells were stained with 4',6-diamidino-2-phenylindole and photographed for fluorescence staining and by phase contrast. The fields shown were chosen to emphasize specific phenotypes and are not necessarily representative of overall frequencies.

complementation data described above, the *skp1-3/skp1-4* heteroallelic diploid exhibits DNA content profiles similar to wild type in logarithmically growing cells at permissive temperature and after shift to the nonpermissive temperature.

Quantitation of cell and nuclear morphology following growth arrest at high temperature also revealed dramatic differences between the *skp1-3* and *skp1-4* mutants. After 3 hr at nonpermissive temperature, *skp1-3* cells are predominantly unbudded with a single DNA mass. A novel class of cells with abnormally elongated buds (21%) is also seen (Figures 4B and 5). After 6 hr at nonpermissive temperature, 57% of *skp1-3* cells exhibit novel cell morphologies, including a multiple

budded phenotype accounting for 37% of the cell population. It appears that *skp1-3* cells do not replicate their DNA (arrest with a G1 DNA content), but continue the budding process. This arrest phenotype is analogous to that seen in a class of *cdc* mutants typified by *cdc4*. In contrast, *skp1-4* cells arrest with a predominant cell morphology indicative of the preanaphase G2/M phase of the cell cycle. After 3 hr at the nonpermissive temperature, 48% of cells were large budded with an undivided nucleus positioned at or near the neck between mother and daughter cells. The majority of cells with this morphology were characterized by DNA at or spanning the neck and a short mitotic spindle. Cells with a short spindle spanning the neck accounted for 16% of the cell

	Temp									n
<b>3 hrs</b>										
<i>SKP1/SKP1</i>	25°C	47	29	6	18	-	-	-	-	316
	37°C	53	25	4	18	-	-	-	-	328
<i>skp1-3/skp1-3</i>	25°C	44	24	17	18	-	-	-	-	422
	37°C	41	15	12	2	21	-	8	-	570
<i>skp1-4/skp1-4</i>	25°C	36	24	15	24	-	1	-	-	420
	37°C	19	17	48*	9	-	5	-	2	450
<i>skp1-3/skp1-4</i>	25°C	42	25	14	19	-	-	-	-	525
	37°C	44	22	17	17	-	-	-	-	541
<b>6 hrs</b>										
<i>SKP1/SKP1</i>	25°C	53	19	9	19	-	-	-	-	304
	37°C	56	18	7	19	-	-	-	-	322
<i>skp1-3/skp1-3</i>	25°C	49	25	13	13	-	-	-	-	323
	37°C	37	4	1	1	20	-	37	-	530
<i>skp1-4/skp1-4</i>	25°C	39	28	14	19	-	-	-	-	321
	37°C	13	10	32	16	2	16	-	11	525
<i>skp1-3/skp1-4</i>	25°C	44	19	20	17	-	-	-	-	430
	37°C	46	16	29	9	-	-	-	-	543

Figure 5. Quantitation of Cell and Nuclear Morphology

*SKP1/SKP1* wild-type, *skp1-3/skp1-3* and *skp1-4/skp1-4* homozygous mutants, and *skp1-3/skp1-4* heteroallelic diploids were grown to logarithmic phase at 25°C and shifted to 37°C for 3 and 6 hr, and nuclear and bud morphology were scored. The criteria used for each morphologic class scored are shown schematically above the columns. The numbers shown represent percentages of the total cells scored (far right column). Asterisk, in this population of cells, 67% had the nucleus at the neck and 33% had the nucleus spanning the neck (n = 203).

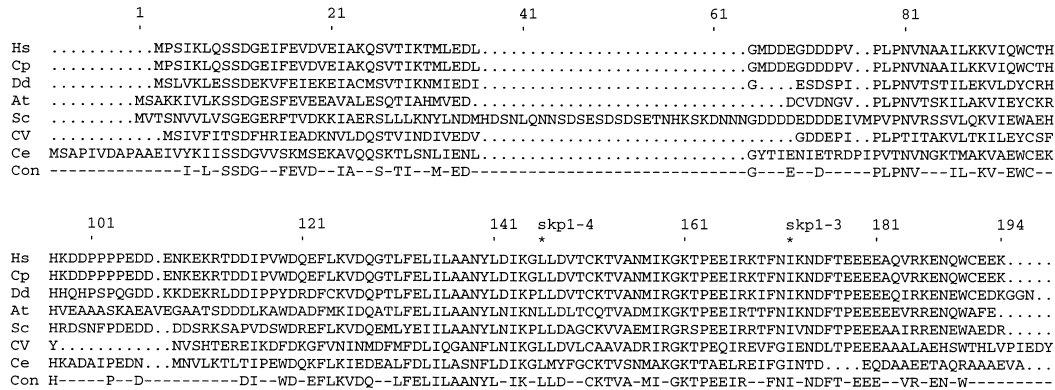


Figure 6. Multiple Protein Sequence Alignments of Skp1p Homologs

The seven Skp1p homologs, from the six species indicated and the chlorella virus genome (PBCV-1), are aligned and ordered by decreasing similarity to the human protein. The consensus is derived from positions that have a 5 out of 7 identical match or better. Hs, *H. sapiens*; Cp, *C. porcellus*; Dd, *D. discoideum*; At, *A. thaliana*; Sc, *S. cerevisiae*; CV, PBCV-1 chlorella virus; Ce, *C. elegans*. Asterisks mark the positions of temperature-sensitive mutations.

population, a morphology rarely seen (<1%) in wild-type cells. Interestingly, at 6 hr a proportion of cells (11%) exhibited a novel multiple budded phenotype (Figures 4B and 5). The *skp1-4* terminal arrest morphology phenotype is therefore like that of *cdc* (at medial nuclear division) and is quite similar to that caused by mutations in the 64 kDa (Cep3p) and 58 kDa (Ctf13p) subunits of CBF3.

We also tested whether cells grown at nonpermissive temperature could recover when shifted back to the permissive temperature. About 60% of *skp1-3* cells were able to recover from the G1 arrest after 6 hr at 37°C. In contrast, only 10% of *skp1-4* cells were able to recover from the G2/M arrest (data not shown).

### Skp1p Is Highly Conserved in Evolution

To date the components of the CBF3 complex in *S. cerevisiae* have not shown highly significant amino acid sequence similarities to other proteins currently present in the public databases. However, when a six-way conceptual translation of the database of expressed sequence tags (dbEST) (Boguski et al., 1994) was searched with the Skp1p amino acid sequence, significant matches were found to ORFs encoded in ESTs from *Arabidopsis thaliana*, *Caenorhabditis elegans*, and *Homo sapiens*. Clones corresponding to the EST sequences were obtained in each case, and the complete sequences of the Skp1p homologs in these three organisms were determined. The human Skp1p homolog sequence is identical to the sequence of p19 (Zhang et al., 1995), which is a component of a cyclin A-CDK2 complex purified from transformed human fibroblasts. Coincidentally, the gene encoding human p19 was also named *SKP1* (for S phase kinase-associated protein). In addition, a search of the nonredundant protein database (NRDB) revealed three sequences with high similarity to Skp1p: OCP-II (Chen et al., 1995), FP21 (West et al., 1995), and an unknown ORF (Lu et al., 1995). OCP-II is a protein found in the organ of Corti in the guinea pig inner ear, and its role in auditory function is unknown. FP21 is a glycoprotein of unknown function in *Dictyostelium* and appears to have an unusual glycosylation

site. The third match is to a predicted 19 kDa protein that corresponds to an ORF of unknown function encoded in the chlorella virus PBCV-1 genome. In addition, Skp1p exhibits weaker homology to the p15 subunit of a transcription factor called SIII (Garrett et al., 1994), which regulates the activity of the RNA polymerase II elongation complex.

The protein sequences of the seven Skp1p homologs were aligned and a consensus generated (Figure 6). A remarkable conservation of this protein has been preserved over the kingdoms, with the highest similarity toward the carboxyl terminus of this protein family. The temperature-sensitive mutations in the *skp1-3* and *skp1-4* alleles are in completely conserved amino acids.

*S. cerevisiae* Skp1p has an apparent 28 amino acid insertion when aligned with the other homologs (residues 37–64). It was possible that these additional residues were important for the specific functions of Skp1p in yeast. An in-frame deletion of the 28 amino acids was constructed (*skp1-Δ2*; see Figure 1E) and shown to rescue a null mutation of *SKP1*. We conclude that the *S. cerevisiae* protein is not a functionally distinct member of the protein family.

### Discussion

CBF3, a multisubunit protein complex that binds the essential CDEIII sequence in centromere DNA, was previously shown to contain three major subunits (58, 64, and 110 kDa in size). We describe the identification of a fourth intrinsic subunit of CBF3, Skp1p, which was isolated by screening for dosage suppressors of a known kinetochore protein mutant. The discovery of Skp1p is of broad significance for several reasons. In addition to its essential structural role at the kinetochore, analysis of *skp1* mutants suggest a role in cell cycle progression at both G2/M and G1/S. Skp1p represents an intrinsic kinetochore protein that is highly conserved in evolutionarily diverse eukaryotes, including yeast and mammals. Yeast Skp1p (suppressor of kinetochore protein) is the homolog of human SKP1 (S phase kinase-associated protein), which is a subunit of a cyclin

A-CDK2 complex purified from cultured mammalian cells. Skp1p may therefore provide a molecular link that facilitates direct interaction between the kinetochore complex and the cell cycle-regulatory machinery.

#### Skp1p Is an Intrinsic Kinetochore Component

Several lines of evidence indicate that Skp1p is a 22.3 kDa subunit of the CBF3 complex. First, increased expression of *SKP1* suppresses the temperature-sensitive lethality and chromosome missegregation phenotype caused by *ctf13-30*, a missense mutation in the 58 kDa subunit of CBF3. Second, *SKP1* is an essential gene, and the temperature-sensitive mutation *skp1-4* causes phenotypes similar to those observed in a *ctf13* mutant, including chromosome missegregation at semi-permissive temperature and a terminal phenotype at nonpermissive temperature indicative of a defect in the G2/M phase of the cell cycle. Finally, antibodies recognizing an epitope-tagged Skp1 protein decrease the electrophoretic mobility of a CBF3-CEN DNA-protein complex formed in vitro.

Within the CBF3 complex, the stoichiometry of Skp1p is apparently equal to Ctf13p. CBF3 has an apparent native molecular mass of 240 kDa, consistent with the original hypothesis that it is a heterotrimeric complex containing one molecule each of the 58, 64, and 110 kDa subunits. The intrinsic mobility shift data (Figure 2A) indicate that Skp1p and Ctf13p are present at an equimolar ratio. Addition of a 60 amino acid segment to either Skp1p or Ctf13p results in an identical mobility change for the CBF3-CEN DNA complex in the absence of added anti-epitope antibody. Exact comigration of these complexes has been confirmed in extended electrophoresis runs (data not shown). This "intrinsic" shift is not subject to variation of epitope accessibility to antibody binding, as might be encountered in a standard supershift assay (as in Figure 2B). CBF3 therefore contains a minimum of four subunits, which are likely to be in an equimolar ratio. The DNase I footprint of CBF3 exhibits asymmetry around the center of the dyad and encompasses 56 bp of DNA (Jehn et al., 1991). It is possible that multiple CBF3 complexes bind to a single CDEIII sequence, especially given the dyad symmetry within the 25 bp CDEIII consensus sequence.

Stemmann and Lechner (1996) have independently identified Skp1p (termed Cbf3d) as a protein that copurifies with Ctf13p (termed Cbf3c) and is required for reconstitution of CBF3 in vitro from purified components. These biochemical data strongly support the conclusion that Skp1p is an essential component of CBF3. Furthermore, as these authors point out, the copurification of Skp1p (Cbf3d) with Ctf13p (Cbf3c) through a series of chromatographic steps provides evidence that these proteins directly interact. Our genetic suppression data provide in vivo evidence for protein-protein interaction: increased expression of *SKP1* suppresses a *ctf13* mutation and, reciprocally, increased expression of *CTF13* suppresses the *skp1-4* mutation.

#### Skp1p Is an Evolutionarily Conserved Kinetochore Component

The identification and sequence analysis of homologs of the *S. cerevisiae SKP1* gene from *A. thaliana*, *C. elegans*,

and *H. sapiens* indicate that this protein is highly conserved (>60% identity in pairwise combinations) throughout the eukaryotic phyla. The human *SKP1* is likely encoded at a single locus, since all 20 examples of *SKP1*-related human EST sequences in dbEST appear to represent overlapping segments of a single mRNA species. Recently, a mouse gene encoding a Skp1p homolog was cloned and related sequences were mapped to two locations in the mouse genome, one corresponding to the cloned gene and the other to a cross-hybridizing sequence (Chen et al., 1995). It is possible that one of these represents a pseudogene, although this point needs clarification. In *C. elegans*, however, *SKP1*-related ESTs were derived from two nonidentical mRNA sequences (only one of which was sequenced to completion and reported here). The human genome may therefore contain a single functional *SKP1* gene or, alternatively, two functional genes, one of which is expressed at low levels and not represented in the cDNA libraries used for EST sequencing. Skp1p homologs present in the public databases include OCP-II (*Cavia porcellus*), FP21 (*Dictyostelium discoideum*), and an uncharacterized ORF from the chlorella virus (PBCV-1) genome; however, very little is known about the function of these three proteins in their respective organisms. The human Skp1p homolog protein sequence is identical to a sequence reported by Zhang et al. (1995) for p19<sup>SKP1</sup>, a protein that copurifies with a p9-p45<sup>SKP2</sup>-cyclin A-CDK2 complex derived from transformed fibroblasts. Although addition of p45 or p45 and p19 to cyclin A-CDK2 complexes had no effect on kinase activity in vitro, microinjection of anti-p45 antibody into cultured cells prevented entry into S phase. Microinjection of anti-p19 antibody had no effect, so there is currently no direct evidence that p19<sup>SKP1</sup> modulates CDK activity either in vitro or in vivo. The G1/S arrest observed in *S. cerevisiae skp1-3* mutants reported here supports the hypothesis that p19 is an essential element of the cyclin A-CDK2 S phase kinase.

#### Skp1p Is Required for Cell Cycle Progression at Both G1/S and G2/M

The distinct sets of phenotypes caused by different temperature-sensitive alleles of *SKP1* suggest two independent functions for the Skp1 protein: one acting at G2/M and the other at G1/S. This is reminiscent of allele-specific G1 or G2/M arrest points caused by different *cdc28* mutant alleles (see Nasmyth, 1993). The G2/M accumulation and chromosome missegregation phenotypes in *skp1-4* mutants are similar to those observed in *ctf13* and *cep3/cbf3c* mutants, both of which carry mutations in CBF3 components. The G2/M delay in cell cycle progression could be due to a physical impediment to chromosome separation or, alternatively, to activation of a cell cycle checkpoint. The identification of *mad* and *bub* mutants that bypass a cell cycle arrest induced by defects in spindle assembly provides strong evidence for a surveillance mechanism that monitors spindle integrity in yeast (Hoyt et al., 1991; Li and Murray, 1991). Two groups have recently shown that these same functions are required for the G2/M delay observed in response to kinetochore defects caused by mutations



in *ctf13* or *CEN* DNA (Wang and Burke, 1995; Pangilinan and Spencer, 1996). It will therefore be of interest to determine the effects of *mad* and *bub* mutants on the *skp1-4*-mediated G2/M delay.

The allele-specific G1/S arrest observed in *skp1-3* mutants appears to be distinct from the G1 arrest observed in *cdc28* mutants. The *skp1-3*-mediated arrest is similar to those exhibited by mutants later in G1. In particular, the multiple budded phenotype is strikingly similar to *cdc4*, *cdc34*, and *cdc53* mutants, which arrest at the G1/S boundary. As noted above, the homology of Skp1p to human p19, which may associate with a cyclin A-CDK2 complex in mammalian cells, is consistent with a regulatory role for entry into S phase. In addition, Bai et al. (1996) provide strong evidence that Skp1p plays a critical role in the transition from G1 to S phase in yeast and mammals. Combined genetic and biochemical experiments show that Skp1p directly interacts with Cdc4p and cyclin F through a novel structural motif. These authors suggest that Skp1p is a component required for ubiquitin-mediated proteolysis of cyclins and CDK inhibitors.

### Skp1p and Cell Cycle Progression

Skp1p is an intrinsic subunit of the kinetochore complex required for assembly of CBF3 on *CEN* DNA (Stemmann and Lechner, 1996; this work). Skp1p also apparently plays an essential regulatory role for entry into S phase (Bai et al., 1996; this work). Temperature-sensitive mutations cause phenotypes consistent with independent roles in kinetochore function at G2/M and cell cycle transition regulation at G1/S (although there is no direct evidence bearing on one or more execution points). Intrallelic complementation supports this idea. What is the relationship between these apparently disparate functions?

One possibility is that defects in Skp1p localized within the kinetochore complex can induce a cell cycle checkpoint at either G2/M or G1/S. Defects in CBF3 structure in *skp1-4* mutants could lead to aberrant or delayed attachment of kinetochores to the mitotic spindle, which in turn would delay the onset of anaphase. Defects in CBF3 structure in *skp1-3* mutants could lead to the G1/S arrest observed by induction of a checkpoint that monitors events at the kinetochore taking place in early S phase. It is known, for example, that centromeres are the earliest-replicating sequences on yeast chromosomes (Ferguson and Fangman, 1992). If *CEN* DNA replication depends on kinetochore structure, failure to complete *CEN* DNA synthesis in a *skp1* mutant could, in theory, preclude subsequent replication of the chromosome.

Alternatively, Skp1p may play spatially distinct but related regulatory roles: one at the kinetochore for progression through mitosis and another elsewhere for entry into S phase. The presence of Skp1p at the kinetochore could provide a direct molecular link to protein complexes (e.g., cyclin-CDKs or other regulatory complexes) whose activities are critical to the metaphase/anaphase transition. Analysis in yeast and higher eukaryotes has provided strong evidence that kinetochores act dynamically to regulate cell cycle progression through mitosis (reviewed by Gorbisky, 1995). A phospho-epitope in animal cells that is present on unat-

tached kinetochores but is extinguished when bipolar attachment is achieved may be a component or target of a kinetochore checkpoint (Campbell and Gorbisky, 1995). As suggested previously (Li and Nicklas, 1995; Gorbisky, 1995), eventual achievement of bipolar attachment could, through mechanical tension on the kinetochore complex, induce a conformational change in a key regulatory protein and thereby regulate the activity of an associated kinase. Though speculative, it is possible that Skp1p may function at the kinetochore to signal completion of metaphase. For example, Skp1p could facilitate docking of a cyclin-CDK complex (or proteins involved in regulating a kinase) at the kinetochore. This specific localization of a regulatory kinase (or other protein complex) may act as a determinant in its activity upon a kinetochore-localized substrate. Perhaps the regulatory capacity of Skp1p has been recruited to the kinetochore by its direct incorporation within the CBF3-*CEN* DNA complex.

### Experimental Procedures

#### Yeast Strains and Media

The *ctf13-30* allele (YPH972) has been previously described (Doherty et al., 1993). The yeast strains and their genotypes used in this study are described in Table 2. The high copy suppression screen was done in YPH972R, a derivative of YPH972 lacking the chromosome fragment. Methods and media for growth and sporulation were as described previously (Rose et al., 1990). Yeast transformations were done by the method described by Ito et al. (1983).

#### High Copy Suppression

A 2 $\mu$  yeast genomic library was prepared from YPH1 (Sikorski and Hieter, 1989) genomic DNA. After partial Sau3A digestion, 6–8 kb of genomic DNA segments was ligated into BamHI-BglIII-restricted pRS202, a derivative of pRS306 that contains, first, the 2 $\mu$  origin and REP3 sequences from Yep24 (HindIII to PstI) inserted into the AatII site and, second, BglIII linkers inserted at the SmaI site of the polylinker. A sample of the library base of 19,500 transformants showed that 32 out of 36 plasmids had average insert sizes of 6–8 kb. All of the recombinants were pooled, and DNA was prepared for transformation into yeast.

Library DNA was transformed into YPH972R, and after 18 hr at room temperature the transformants were shifted to the nonpermissive temperature at 37°C selecting for uracil prototrophy. Approximately 18,300 independent transformants were screened at the nonpermissive temperature, yielding 15 temperature-resistant colonies after 5 days of growth. The library clones were recovered from the yeast strains and retested for the suppression phenotype. Restriction site and Southern blot analysis of the plasmid DNAs indicated that 7 of 15 contained *CTF13* sequences, 3 of 15 contained vector only or an insert that failed to retest, and 5 of 15 contained overlapping segments. The latter class of plasmids were able to suppress the temperature sensitivity of *ctf13-30*, although not as well as wild-type Ctf13p.

#### Molecular Characterization of *SKP1*

The library clone (3B) chosen for further characterization contained a 6.3 kb insert of genomic DNA (Figure 1A). A 1.68 kb SpeI-PvuII fragment was subcloned and shown to suppress the temperature sensitivity of a *ctf13-30* mutant. Fill-in of a unique NsiI site with T<sub>4</sub>DNA polymerase (New England Biolabs) abolished the suppressing activity. A series of subclones and deletion derivatives were sequenced by standard methods (Sanger et al., 1977; Hattori and Sakaki, 1986; Smith et al., 1986; McCombie et al., 1992). *CEN* vector derivatives containing the 1.68 kb SpeI-PvuII region were constructed by insertion into the SpeI and SmaI polylinker sites of pRS vectors (Sikorski and Hieter, 1989), resulting in two constructs, pRS315SKP and pRS316SKP. These *CEN* vector recombinants were able to suppress the temperature sensitivity of *ctf13-30*, but not to the extent of the 2 $\mu$  library clone 3B. Insertion into the *SKP1* ORF

Table 2. Strains Used in This Study

<i>S. cerevisiae</i> Strain S288C Derivatives	Genotype	Reference
YPH972R <sup>a</sup>	<i>Mat<math>\alpha</math> ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>1 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 ctf13-30</i>	Doheny et al., 1993
YPH975R <sup>a</sup>	<i>Mat<math>\alpha</math> ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>1 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 ctf13<math>\Delta</math>1::HIS3 pRS316/CTF13</i>	Doheny et al., 1993
YPH985	<i>Mata/Mat<math>\alpha</math> ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-<math>\Delta</math>63/trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200/his-<math>\Delta</math>200 leu2-<math>\Delta</math>1/leu2-<math>\Delta</math>1 CFIII (CEN3.L.YPH 983) HIS3 SUP11</i>	This study
YPH1015	<i>Mata ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 CFIII (CEN3.L.YPH983) HIS3 SUP11</i>	This study
YPH1096	<i>Mata ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 CFIII (CEN3.L.YPH983) HIS3 SUP11 pRS316SKP1</i>	This study
YPH1099	<i>Mata ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 <i>skp1<math>\Delta</math>1::TRP1</i> CFIII (CEN3.L.YPH983) HIS3 SUP11 pRS316SKP1</i>	This study
YPH1161	<i>Mata ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 <i>skp1<math>\Delta</math>1::TRP1 skp1-4::LEU2</i> CFIII(CEN3.L.YPH983) HIS3 SUP11</i>	This study
YPH1172	<i>Mata ura3-52 lys2-801 ade2-101 trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 <i>skp1<math>\Delta</math>1::TRP1 skp1-3::LEU2</i> CFIII(CEN3.L.YPH983) HIS3 SUP11</i>	This study
YPH1197	<i>Mata/Mat<math>\alpha</math> ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-<math>\Delta</math>63/trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200/his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1/leu2-<math>\Delta</math>1 <i>skp1-3::LEU2/skp1-3::LEU2</i> CFIII (CEN3.L.YPH983) HIS3 SUP11</i>	This study
YPH1201	<i>Mata/Mat<math>\alpha</math> ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-<math>\Delta</math>63/trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200/his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1/leu2-<math>\Delta</math>1 <i>skp1-4::LEU2/skp1-4::LEU2</i> CFIII (CEN3.L.YPH983) HIS3 SUP11</i>	This study
YPH1208	<i>Mata/Mat<math>\alpha</math> ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-<math>\Delta</math>63/trp1-<math>\Delta</math>63 his3-<math>\Delta</math>200/his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1/leu2-<math>\Delta</math>1 <i>skp1-3::LEU2/skp1-4::LEU2</i> CFIII (CEN3.L.YPH983) HIS3 SUP11</i>	This study

<sup>a</sup> These strains are mitotic segregants that have lost the marker chromosome fragment (and therefore form red colonies).

of a 1.8 kb BglII fragment containing the *TRP1* gene was accomplished by blunt-end ligation into the NsiI site. One-step gene replacement was accomplished by excision of a NotI-XhoI fragment and transformation into YPH985 selecting for Trp<sup>+</sup> transformants. The integration of the *TRP1* marker into the genome was confirmed by Southern blot analysis of restricted genomic DNA. Trp<sup>+</sup> diploids were sporulated, and 20 tetrads dissected resulted in 2+ : 2- segregation for viability. All viable spores were Trp<sup>-</sup>, demonstrating that *SKP1* was an essential gene.

*SKP1* was physically mapped to chromosome IV by hybridization of a radioactive <sup>32</sup>P-labeled (Feinberg and Vogelstein, 1984) EcoRI-NotI (polylinker) fragment isolated from 3B to filters containing overlapping  $\lambda$  and cosmid clones of the *S. cerevisiae* genome (L. Riles and M. Olson). This placed *SKP1* on the right arm of chromosome IV, between *gcn2* and *trp4*.

The *skp1- $\Delta$ 1::TRP1* allele is a deletion of the entire *SKP1* ORF between the PstI and Eco57I sites and replacement with vector and *TRP1* sequences using one-step gene replacement (Figure 1B). This deletion extends from 100 bp upstream of the start of *SKP1* to 20 bp upstream of the stop codon. The integration vector, pRS304  $\Delta$ SKP1, was constructed by inserting the ~1.3 kb PstI (polylinker)-PstI fragment and the 1.16 kb Eco57I-ClaI fragment from the original library clone 3B into the PstI site and the ClaI-KpnI (blunt) site of pRS304 (Sikorski and Hieter, 1989), respectively. pRS304  $\Delta$ SKP1 was linearized with EcoRI and transformed into YPH1096 selecting for Trp<sup>+</sup> transformants. Transformants were streaked on 5-fluoroorotic acid (FOA) (Boeke et al., 1987) to determine whether the integration took place in the genome or on the Ura<sup>-</sup> plasmid, pRS316SKP1. Transformants that failed to grow on FOA were selected, and Southern blot analysis of genomic DNA was performed to confirm the integration and removal of the *SKP1* ORF. YPH1099 is the *skp1* null strain generated in this way.

#### Biochemical Analysis

The plasmid containing the *GAL1* promoter-E1 epitope fused to *SKP1*, p415GEU1SKP, was constructed from the plasmid p415GEU1, a derivative of p414GEU1 (Doheny et al., 1993), which replaced the *TRP1* gene with the *LEU2* gene using PvuII-PvuII sites in the vector sequence (Sikorski and Hieter, 1989). A PCR strategy was used to place the E1 epitope in-frame with the second codon of the *SKP1* ORF using an engineered XhoI cloning site and a downstream genomic EcoRI site (see Figure 1A). To avoid possible PCR errors, wild-type genomic sequence from NsiI to PvuII replaced the PCR-generated sequence. When transformed into the *skp1* deletion strain, p415GEU1SKP rescued growth on galactose-containing, but not dextrose-containing, media. The untagged construct was prepared by cloning the XhoI-PvuII fragment from p415GEU1SKP into p415GEU2 (J. Kroll, unpublished data) at the XhoI-SmaI sites in the polylinker. When transformed into the *skp1* deletion strain, p415GEU2SKP rescued growth on both galactose- and dextrose-containing media, presumably because of low level expression under glucose-repressing conditions.

The HA epitope was fused to *SKP1* in pAD5 (Neiman et al., 1993), which is a 2 $\mu$ -LEU2 plasmid that carries an *ADH1* promoter, an ATG, and the Lerner HA epitope (Field et al., 1988) followed by a Sall cloning site. A 1.3 kb XhoI-SacI fragment from p415GEU1SKP was cloned into the Sall-SacI site in the polylinker of pAD5, resulting in pAD5SKP. This HA-tagged construct was able to rescue viability in the *skp1* deletion strain.

Extracts were prepared for biochemical analysis of CBF3-CEN DNA complexes according to the methods described in Doheny et al. (1993). The DNA fragment containing CDEIII used in the gel mobility shift assay was the same as reported previously (Doheny et al., 1993) except it was labeled by a different method. The plasmid pSF262a (a gift from P. Sorger) allows excision of the CDEIII region with HindIII-EcoRI, resulting in a 100 bp fragment that was labeled

using Taq polymerase according to the method described previously (Niedenthal and Hegemann, 1993).

#### Generation and Integration of Temperature-Sensitive Alleles

Synthetic oligonucleotides were synthesized corresponding to sequences approximately 250 bp on either side of the polylinker of pRS316. They served as primers for a PCR using pRS316SKP as a template following conditions for mutagenesis as described by Muhrad et al. (1992). Approximately 10 ng of template was amplified under standard PCR conditions (Perkin Elmer) except the concentration of MgCl<sub>2</sub> was increased to 3 mM and the dNTP pool was reduced to 60 μM. Approximately 500 ng of mutagenized PCR product was cotransformed into YPH1099 with 500 ng of XhoI-linearized pRS315 and 5 μg of herring sperm DNA (Sigma). Transformants were replica plated twice to medium containing FOA to ensure eviction of pRS316SKP. Of the 1300 transformants screened by this method, 16% failed to grow on FOA, suggesting knockouts of *SKP1* or reclosing of the gapped vector. There was an approximately 4-fold stimulation of transformants with the addition of PCR product as compared with gapped vector only. It was also observed that approximately 80% of the gapped vector only transformants failed to grow on FOA. Transformants that grew presumably recombined with pRS316SKP. The transformants were then replica plated in parallel and grown at 25°C and 37°C. Four temperature-sensitive alleles, *skp1-1*, *skp1-2*, *skp1-3*, and *skp1-4*, were isolated, and the mutagenized plasmids were recovered and retested for sensitivity at 37°C. The nucleotide sequence of each allele was done to identify the mutations (Smith et al., 1986; McCombie et al., 1992), and an alignment of the sequences revealed that the mutations fell mostly in the carboxyl terminus (see Table 1).

Two of the temperature-sensitive alleles, *skp1-3* and *skp1-4*, were integrated into the genome at *leu2-Δ1* on chromosome III using a *LEU2* derivative of p679 (Doheny et al., 1993). The mutagenized plasmid DNA was excised from the pRS315SKP derivative as a 1.6 kb SpeI (blunt)-HindIII (polylinker) and inserted into a SmaI-HindIII polylinker site of p679LEU2, resulting in BPH650 (*skp1-3*) and BPH651 (*skp1-4*). Transformation of these two constructs linearized with NotI directed the γ-integration of the temperature-sensitive alleles to *leu2-Δ1* located either on chromosome III or the artificial chromosome III fragment in YPH1099. Independent transformants were grown in the presence of FOA to select against the wild-type *SKP1* and then tested for temperature sensitivity. Transformants that grew at the permissive temperature but failed to grow at the nonpermissive temperature were prepared for analysis by orthogonal field alteration gel electrophoresis (OFAGE) according to the methods described by Carle and Olson (1984). The genomic location of the temperature-sensitive allele was accomplished by hybridization of a 1.3 kb PstI (genomic)-PstI (polylinker) containing *SKP1* from pRS315SKP to a Southern blot of the OFAGE gel (data not shown). The genomic integration of *skp1-3* was confirmed in YPH1172 and that of *skp1-4* in YPH1161.

Homozygous and heterozygous *skp1* diploids were generated from meiotic sister spores obtained from dissection of tetrads from backcrosses of YPH1172 and YPH1161 to YPH500 (Sikorski and Hieter, 1989; see Table 2).

#### Internal Deletion of *SKP1* ORF

The alignment of *S. cerevisiae* to conserved homologs in other species (see Figure 6) defined a region of 28 amino acids unique to *S. cerevisiae*. Fortunately, a genomic NsiI site was at the junction. This region of nonhomology was removed from the *SKP1*-coding region by a PCR strategy using a synthetic oligonucleotide, PH139, which included a unique NsiI cloning site in-frame with the sequence 5'-GGTGACGACGACGATGAGGATG-3' located at the end of the 28 amino acid region to be removed. An ~980 bp PCR product generated from PH139 and a primer in the vector sequence of pRS316SKP was restricted with NsiI-XhoI and cloned into the NsiI (genomic)-XhoI (polylinker) of pRS315SKP, resulting in pRS315 *skp1-Δ2*. This construct rescued the deletion strain YPH1099.

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#### GenBank Accession Numbers

The accession numbers for the SKP1 cDNA sequences reported in this paper are U43179 (*S. cerevisiae*), U60980 (*C. elegans*), and U60981 (*A. thaliana*).