Yeast Ubiquitin-like Genes Are Involved in Duplication of the Microtubule Organizing Center

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Abstract. *KAR1* is required for duplication of the *Saccharomyces cerevisiae* microtubule organizing center, the spindle pole body (SPB) (Rose, M.D., and G.R. Fink, 1987. *Cell.* 48:1047–1060). Suppressors of a *kar1* allele defective for SPB duplication were isolated in two genes, *CDC31* and *DSK2* (Vallen, E.A., W.H., M. Winey, and M.D. Rose. 1994. *Genetics.* 137:407–422). To elucidate the role of *DSK2* in SPB duplication, we cloned the gene and found it encodes a novel ubiquitin-like protein containing an NH_2 terminus 36% identical to ubiquitin. The only other known yeast ubiquitin-like protein is encoded by the nucleotide excision repair gene *RAD23* (Watkins, J.F., P. Sung, L. Prakash, and S. Prakash. 1993. *Mol. Cell. Bio.* 13:7757–7765). Unlike ubiquitin, the NH_2-terminal domain of Dsk2p is not cleaved from the protein, indicating that Dsk2p is not conjugated to other proteins. Although the *DSK2-1* mutation alters a conserved residue in the Dsk2p ubiquitin-like domain, we detect no differences in Dsk2p or Cdc31p stability. Therefore, *DSK2* does not act by interfering with ubiquitin-dependent protein degradation of these proteins. Although *DSK2* is not essential, a strain deleted for both *DSK2* and *RAD23* is temperature sensitive for growth due to a block in SPB duplication. In addition, overexpression of *DSK2* is toxic, and the *DSK2-1* allele causes a block in SPB duplication. Therefore, *DSK2* dosage is critical for SPB duplication. We determined that *CDC31* gene function is downstream of *DSK2* and *KAR1*. Dsk2p is a nuclear-enriched protein, and we propose that Dsk2p assists in Cdc31 assembly into the new SPB.

THE eukaryotic microtubule cytoskeleton is organized and regulated by microtubule organizing centers (MTOC)[1]. The MTOC of the yeast *Saccharomyces cerevisiae* (called the spindle pole body [SPB]) is similar to the centrosome of higher eukaryotes and is essential for microtubule-dependent processes such as mitosis and meiosis (for review see Winey and Byers, 1993). In addition to their similar functions, the centrosome and SPB are subject to similar cell cycle regulation because they must be duplicated once per cell cycle to form a mitotic spindle. However, despite the functional equivalence between the yeast SPB and the mammalian centrosome, the morphology between the structures is different.

The SPB is a trilaminar structure containing a central plaque, which is physically embedded in the nuclear envelope and inner and outer plaques that nucleate the nuclear and cytoplasmic microtubules, respectively (Byers and Goetsch, 1974, 1975). A membranous structure, the half-bridge, extends from the central plaque within the nuclear envelope. The entire SPB must faithfully duplicate once per cell cycle to form the poles of the mitotic spindle. At the beginning of the cell cycle, a satellite structure that is thought to be the precusor to the new SPB forms on the outer face of the half-bridge of the sole SPB. The next step is the formation of a new SPB, which is inserted into the nuclear envelope, resulting in adjacent SPBs connected by a complete bridge structure. The bridge is severed in a step that involves kinesin-like motor proteins (Hoyt et al., 1992; Roof et al., 1992), which act to separate the SPBs to opposite sides of the nucleus to form the poles of the mitotic spindle. After mitosis and cytokinesis, the resulting daughter cells each contain a single SPB. Although SPB duplication once per cell cycle is essential for bipolar mitotic spindle formation, very little is known about the regulation of this essential process.

The Kar1 protein, a component of the SPB (Vallen et al., 1992*b*; Spang et al., 1995), is essential for SPB duplication during the cell cycle (Rose and Fink, 1987). To identify additional proteins involved in SPB duplication, suppressors of a *kar1* allele defective for SPB duplication, *kar1Δ17*, were isolated (Vallen et al., 1992*a*, 1994). The

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1. *Abbreviations*: DAPI, 4',6-diamidino-2-phenylindole; 5-FOA, 5-fluoroorotic acid; GST, glutathione-*S*-transferase; MTOC, microtubule organizing center; ORF, open reading frame; SC, synthetic complete medium; SPB, spindle pole body; ub-like, ubiquitin-like; YPD, yeast extract/peptone/dextrose medium.

Table I. Strains Used in This Study

Strain	Genotype	Source
MS10	*MAT***a** *ura3-52 leu2-3,112 ade2-101*	
MS1554	*MAT***a** *ura3-52 leu2-3,112 ade2-101 his3Δ200*	
MS2082	*MATα ura3-52 leu2-3,112 ade2-101 kar1Δ17*	*
MS2373	*MAT***a** *ura3-52 leu2-3,112 trp1Δ1 kar1Δ17;* [pMR76]	
MS2374	*MATα ura3-52 leu2-3112 trp1Δ1 ade2-101 kar1Δ17;* [pMR76]	
MS2645	*MAT***a** *ura3-52 leu2-3,112 ade2-101 kar1Δ2, DSK2-1*	
MS2646	*MATα ura3-52 leu2-3,112 trp1Δ1 ade2-101 kar1Δ2 DSK2-1*	
MS3134	*MATα ura3-52 leu2-3,112 ade2-101 kar1Δ17 DSK2-1*	‡
MS3137	*MATα ura3-52 leu2-3,112 ade2-101 kar1Δ17 CDC31-16*	‡
MS3145	*MATα ura3-52 leu2-3,112 ade2-101 kar1Δ17 DSK2-1*	
MS3486	*MAT***a** *ura3-52 ade2-101 lys2-801 trp1Δ1 CDC31-16;* [pMR2745]	
MS3487	*MAT***a** *ura3-52 ade2-101 lys2-801 trp1Δ1 CDC31-16;* [pMR1872]	
MS3574	*MAT***a** *ura3-52 leu2-3,112 ade2-101 dsk2Δ::LEU2*	
MS3664	*MAT***a** *ura3-52 leu2-3,112 trp1Δ1 kar1Δ17 dsk2Δ::LEU2*	
MS3759	*MAT***a** *ura3-52 leu2-3,112 ade2-101 his3Δ200 DSK2-1*	
MY424	*MAT***a** *ura3-52 leu2,3-112 his4-519 ade1-100*	
MY2259	*MATα ura3 trp1 his7 cdc31-1*	§
MY2260	*MATα ura3 leu2 trp1 ade2 cdc31-2*	§
MY2261	*MATα ura3 ade met2 cdc31-5*	§
MY3316	*MAT***a** *ura3-52 leu2,3-112 his4-519 ade1-100;* [pMR2745]	
MY3317	*MAT***a** *ura3-52 leu2,3-112 his4-519 ade1-100;* [pMR2757]	
MY3320	*MATα ura3 trp1 his7 cdc31-1;* [pMR1872]	
MY3321	*MATα ura3 trp1 his7 cdc31-1;* [pMR2745]	
MY3324	*MAT***a** *ura3-52 leu2,3-112 his4-519 ade1-100;* [pMR1872]	
MY3328	*MATα ura3 ade met2 cdc31-5;* [pMR1872]	
MY3329	*MATα ura3 ade met2 cdc31-5;* [pMR2745]	
MY3334	*MATα ura3 leu2 trp1 ade2 cdc31-5;* [pMR2745]	
MY3335	*MAT***a** *ura3 leu2 trp1 ade2 cdc31-2;* [pMR1872]	
MY3516	*MAT***a**/*MATα lys2-801/+ trp1Δ63 leu2/leu2 ura3-52/ura3-52;* [pMR613]	
MY3522	*MAT***a**/*MATα lys2-801/+ trp1Δ63/trp1Δ63 leu2/leu2 ura3-52/ura3-52;* [pMR2905]	
MY3523	*MAT***a**/*MATα lys2-801/+ trp1Δ63 leu2/leu2 ura3-52/ura3-52;* [pMR2906]	
MY3545	*MATα ura3-52 lys2 ade2-1 leu2Δ his3Δ200 rad23Δ::URA3*	‖
MY3573	*MATα ura3-52 leu2 his3Δ200 ade2 dsk2Δ::LEU2 rad23Δ::URA3*	
MY3587	*MATα ura3-52 leu2 his3 ade2 rad23Δ::URA3*	
MY3588	*MAT***a** *ura3-52 trp1 ade2 his3 dsk2Δ::LEU2*	
MY3589	*MAT***a** *ura3-52 leu ade2*	
MY3592	*MATα ura3-52 leu2 his3Δ200 ade2 dsk2Δ::LEU2 rad23Δ*	
MY3602	*MATα ura3-52 leu2 his3Δ200 ade2 dsk2Δ::LEU2 rad23Δ;* [pMR2032]	
MY3603	*MATα ura3-52, leu2 his3Δ200 ade2 dsk2Δ::LEU2 rad23Δ;* [pMR2223]	
MY3604	*MATα ura3-52 leu2 his3Δ200 ade2 dsk2Δ::LEU2 rad23Δ;* [pMR1868]	
MY3714	*MATα ura3-52 leu2,3-112 his3 ade2 rad23Δ kar1Δ17;* [pMR76]	
MY3715	*MATα ura3-52 leu2,3-112 his3 ade2 rad23Δ kar1Δ17*	
MY3740	*MAT***a** *ura3-52 leu2,3-112 ade2-101 kar1Δ17 DSK2+ RAD23+*	
MY3741	*MATα ura3-52 leu2,3-112 lys trp1Δ63 ade2-101 kar1Δ17 dsk2Δ::LEU2 rad23Δ*	
MY3742	*MATα ura3-52 leu2,3-112 lys his ade2-101 kar1Δ17 DSK2+ rad23Δ*	
MY3743	*MAT***a** *ura3-52 leu2,3-112 ade 2-101 his kar1Δ17 dsk2Δ::LEU2 RAD23+*	
MY3752	*MAT***a** *ura3-52 leu2,3-112 ade2-101 kar1Δ17 DSK2+ RAD23+*; [pMR2223]	
MY3753	*MAT***a** *ura3-52 leu2,3-112 ade2-101 kar1Δ17 DSK2+ RAD23+*; [pMR2032]	
MY3754	*MAT***a** *ura3-52 leu2,3-112 ade2-101 kar1Δ17 DSK2+ RAD23+*; [pMR2745]	
MY3755	*MAT***a** *ura3-52 leu2,3-112 ade2-101 kar1Δ17 DSK2+ RAD23+*; [pMR1868]	
MY3756	*MATα ura3-52 leu2,3-112 lys trp1Δ63 ade2-101 kar1Δ17 dsk2Δ::LEU2 rad23Δ;* [pMR2223]	
MY3757	*MATα ura3-52 leu2,3-112 lys trp1Δ63 ade2-101 kar1Δ17 dsk2Δ::LEU2 rad23Δ;* [pMR2032]	
MY3758	*MATα ura3-52 leu2,3-112 lys trp1Δ63 ade2-101 kar1Δ17 dsk2Δ::LEU2 rad23Δ;* [pMR2745]	
MY3759	*MATα ura3-52 leu2,3-112 lys trp1Δ63 ade2-101 kar1Δ17 dsk2Δ::LEU2 rad23Δ;* [pMR1868]	
MY4240	*MAT***a**/*MATα ura3-52/ura3-52 leu2/leu2 his3/HIS3 lys2/LYS2 TRP1/trp1Δ1 ade2/ade2 rad23Δ/rad23Δ::URA3 dsk2Δ::LEU2/dsk2Δ::LEU2*	

All strains designated MS are isogenic with strain S288c. Unless noted otherwise, all strains were constructed for this study or are Rose lab strains.
*Vallen et al., 1992a.
‡Vallen et al., 1994.
§B. Byers, University of Washington, Seattle, WA.
‖S. Prakash, University of Texas, Galveston, TX.

dominant extragenic suppressors defined two linkage groups, *CDC31* and *DSK2*. *CDC31* encodes a calcium-binding protein (Baum et al., 1986) most homologous to centrin/caltractin, a component of the *Chlamydomonas reinhardtii* MTOC (Huang et al., 1988*a*,*b*; Salisbury et al., 1988). This protein is a conserved component of the MTOC in yeast (Spang et al., 1993; Biggins and Rose, 1994) and other eukaryotic organisms (e.g., Lee and Huang, 1993). The *KAR1* and *CDC31* genes display a number of genetic interactions (Vallen et al., 1994), and the protein products interact directly in vitro (Biggins and Rose, 1994; Spang et al., 1995). Although Cdc31p localizes to the SPB in wild-type cells, it was mislocalized in the *kar1Δ17* mutant (Biggins and Rose, 1994; Spang et al., 1995). Suppressor alleles of *CDC31* restored SPB localization of Cdc31p, suggesting that Kar1p's mitotic function is to recruit and maintain Cdc31p at the SPB. Since some of the suppressors completely bypass the requirement for *KAR1*, suppression cannot be due to an altered interaction between Cdc31p and Kar1p. Therefore, it is most likely that the *CDC31* suppressors have an increased interaction with another SPB component and no longer require Kar1p for SPB localization.

Two independent alleles of *DSK2* isolated as *kar1Δ17* suppressors also bypass the essential function of *KAR1* (Vallen et al., 1994). The Dsk2p suppressor proteins might directly interact with Cdc31p to localize it to the SPB in a *kar1* deletion strain, or they might act indirectly to bypass the *KAR1* function. To elucidate the role of the *DSK2* suppressors in SPB duplication, we cloned *DSK2* and found it encodes a novel ubiquitin-like protein.

Ubiquitin is a highly conserved essential 76–amino acid protein that is posttranslationally conjugated to substrates to modify their function (for reviews see Jentsch, 1992; Ciechanover, 1994). Ubiquitin is synthesized as a fusion protein in which the first 76 amino acids encode ubiquitin and the remainder encodes either other ubiquitins or unrelated proteins that are often involved in protein synthesis. Cellular hydrolases (E1 enzymes) cleave the primary translation product to generate free ubiquitin, which can then be conjugated to substrate proteins by ubiquitin-conjugating enzymes (E2 enzymes). Substrates can be monoubiquitinated, which may change function, or polyubiquitinated, which targets them for degradation via the proteasome. Conjugation sometimes requires the function of a third protein (E3 ligase).

The ubiquitin-like proteins are a growing family of proteins that contain NH_2-terminal domains homologous but not identical to true ubiquitin. Although these proteins have been identified in many organisms, the functions of almost all of them are unknown. One of the major classes of ubiquitin-like genes is involved in DNA repair and includes the yeast gene *RAD23* (Watkins et al., 1993) and its human homologues, the XP-C genes (Masutani et al., 1994). *RAD23* was the first ubiquitin-like gene identified in yeast and was originally isolated in a screen for UV-sensitive mutants. A *rad23Δ* does not exhibit growth defects but displays an increased sensitivity to UV light (Watkins et al., 1993). Rad23p is a highly stable nuclear protein, and its function in DNA repair may be to facilitate the interaction between the nucleotide excision repair protein Rad14 and the TFIIH subunit of RNA polymerase (Guzder et al., 1995). Here we show that *DSK2* also encodes a ubiquitin-like protein in which the first 76 amino acids are significantly homologous to ubiquitin and the remainder of the protein does not exhibit homology to any known proteins. In addition, we show that *DSK2+* gene function is required with *RAD23* function for SPB duplication, and we present models for the potential role of the *DSK2+* gene.

Materials and Methods

Microbial Techniques and Yeast Strain Constructions

Yeast media and genetic and microbial techniques were essentially as described in Rose et al. (1990). Bacterial media was as described in Sambrook et al. (1989), and bacterial strains XL1-Blue and BL21 were used for all bacterial manipulations. 5-fluoroorotic acid (5-FOA) was used to select Ura- segregants as described by Boeke et al. (1984).

All yeast strains used are listed in Table I. Yeast strains were constructed by standard genetic techniques. Diploids were selected on the appropriate selective media at 23°C and subsequently sporulated at 23°C. In crosses containing the *kar1Δ17* allele, a *KAR1+ URA3* plasmid was present to prevent increased ploidy before the cross. After the tetrads were dissected, Ura- derivatives were selected on 5-FOA (Boeke et al., 1984).

A two-step gene replacement method (Rothstein, 1991) was used to construct a *dsk2Δ* strain (MS3574). Plasmid pMR2860 was cut with MscI and transformed into haploid (MS10) and diploid (MS810) strains. Transformants were selected on synthetic complete (SC)-leu media at 23°C and subsequently streaked onto 5-FOA to select -Ura recombinants. To assay for the presence of the *dsk2Δ*, PCR analysis was performed with a primer within the *LEU2* gene (5′ ACA/CCA/TAC/CCA/TCG/TCT 3′; courtesy of Scott Holmes, Princeton University, NJ) and a *DSK2* primer (5′ GGG/GAG/ACC/TTA/TTT 3′). PCR analysis confirmed the presence of the *dsk2Δ* in the haploid strain, indicating that *DSK2* is not required for cell viability. The deletion was also confirmed by Western blotting of *dsk2Δ* strain (MS3574) extract with anti-Dsk2p antibodies that confirmed the absence of Dsk2p.

A *DSK2-1 KAR1+* strain was constructed by crossing a *DSK2-1 kar1Δ2* strain (MS2646) to a *DSK2+ KAR1+* strain (MS1554). A nonparental ditype from this cross was backcrossed to confirm the genotype as *DSK2-1 KAR1+*. To generate a *dsk2Δ rad23Δ* strain, a *rad23Δ* strain (MY3545) marked with *URA3* was crossed to a *dsk2Δ* strain (MS3574) marked with *LEU2*. To allow further genetic manipulations with the *URA3* marker, a Ura- *dsk2Δ rad23Δ* strain (MY3592) was generated by selecting 5-FOA–resistant segregants of one of the Ura+ Leu+ spores. This was possible because the *rad23Δ* marked with *URA3* was flanked by direct repeats (Watkins et al., 1993). To generate a *dsk2Δ kar1Δ17* strain, a *DSK2+ kar1Δ17* strain (MS2374) was crossed to a *dsk2Δ KAR1+* strain (MS3574). To generate a *CDC31-16 dsk2Δ kar1Δ17* strain, a *dsk2Δ kar1D17* strain (MS3664) was crossed to a *CDC31-16 kar1Δ17* strain (MS3137). A *rad23Δ kar1Δ17* strain was generated by crossing a *dsk2Δ rad23Δ* strain (MY3592) to a *kar1Δ17* strain (MS2373). To generate a *dsk2Δ rad23Δ kar1Δ17* strain, a *rad23Δ kar1Δ17* strain (MY3714) was crossed to a *dsk2Δ kar1Δ17* strain (MS3664).

To test suppression of *rad23Δ dsk2Δ* temperature sensitivity by *CDC31-16* and high copy *CDC31+*, plasmids pMR2023 (2 μm, *CDC31+*), pMR2223 (2 μm, *CDC31-16*), and pMR1868 (pRS416; Sikorski and Hieter, 1989) were transformed into MY3592 to generate strains MY3602 (pMR2032), MY3603 (pMR2223), and MY3604 (pMR1868). Serial dilutions of the transformants were tested for growth at 37°C and determined that *CDC31-16* and high copy *CDC31+* suppress the temperature sensitivity of the *rad23Δ dsk2Δ* strain. Strains MY3752-MY3763 were generated and tested for growth at 33°, 35°, and 37°C the same way by transforming MY3741.

Growth curves on strains MY3522 and MY3523 were performed by growing them in SC-ura media with 2% raffinose at 30°C to early logarithmic phase. The cultures were split in half, and galactose or glucose was added to each half to 2% final concentration. Aliquots were taken every hour upon galactose or glucose addition, and serial dilutions were plated on SC-ura glucose plates. The viability was calculated as the number of viable cells per ml at each time point. Growth curves on strains MY3587, MY3588, MY3589, and MY3592 were performed the same way, except the strains were grown at 30°C in yeast extract/peptone/dextrose (YPD)

media to early logarithmic phase and then shifted to 37°C. Cells were harvested every hour and plated on YPD plates at 30°C.

Plasmid Constructions

All restriction enzymes used in this study were obtained from New England Biolabs (Beverly, MA) and were used according to the supplier specifications. All PCR primers were synthesized at the Princeton University sequencing facility. All cloning techniques were performed according to Sambrook et al. (1989).

DSK2 subclones were obtained by isolating restriction fragments (shown in Fig. 1 *A*) from the smallest suppressing plasmid (pMR3165) and by ligating them into pRS416 (Sikorski and Hieter, 1989) or YCp50 (Rose et al., 1987). To determine which open reading frame (ORF) suppressed *kar1Δ17*, the *URA3* gene was isolated on a 1.1-kb HIII fragment and filled in with Klenow. This was ligated into pMR2719, which had been linearized with NcoI and filled in with Klenow to generate pMR2718 that disrupts ORF2 (shown in Fig. 1 *B*).

Glutathione-*S*-transferase (GST)–Dsk2p bacterial expression plasmids were generated by PCR amplification of the *DSK2+* and *DSK2-1* genes using primers 5′GGC/AAA/TAA/GAC/ GGA/TCC/AAG/ACA/CCG 3′ and 5′ GAT/ATT/ATT/GAT/GGA/TCC/ATG/TAA/TAC 3′. These primers have BamHI sites engineered to allow subsequent digestion and ligation of the PCR products such that the entire *DSK2* gene will be expressed. PCR was performed on plasmids pMR2745 (*DSK2-1*) and pMR2757 (*DSK2+*), and the 1.8-kb product was digested with BamHI and ligated into the BamHI site of pGEX-KT (courtesy of Steve Haney, Princeton University, NJ) to generate pMR2863 (GST-Dsk2-1p) and pMR2862 (GST-Dsk2p). These plasmids were transformed into BL21 to generate strains MR2865 (pMR2862) and MR2863 (pMR2863), which were used for Dsk2p expression and purification to generate antibodies. To generate GST-Dsk2p ubiquitin-like domain fusions, the first 80 amino acids of Dsk2p were cloned into the pGEX-2T vector (courtesy of Steve Haney). PCR was performed on pMR2757 (*DSK2+*) and pMR2745 (*DSK2-1*) using primers 5′GGC/AAA/TAA/GAC/GGA/TCC/AAG/ACA/CCG 3′ and 5′ GGG/GAA/TCC/GGC/GGT/TTG/TGG/TTT/GGG 3′. The 275-bp amplified DNA product was digested with BamHI and EcoRI (sites were engineered into the PCR primers) and cloned into pGEX-2T cut with BamHI/EcoRI (courtesy of Steve Haney) to generate plasmids pMR2908 (GST-Dsk2p ubiquitin-like domain) and pMR2909 (GST-Dsk2-1p ubiquitin-like domain). These plasmids were transformed into BL21 to generate strains MR2910 (pMR2908) and MR2911 (pMR2909) for expression and purification of the recombinant proteins.

The full-length *DSK2+* and *DSK2-1* PCR products described above were cloned into centromere-based vector pRS414 (Sikorski and Hieter, 1989) at the BamHI site and checked for suppression of *kar1Δ17* to confirm the presence or absence of the *DSK2-1* mutation. These plasmids, pMR2838 (*DSK2+*) and pMR2859 (*DSK2-1*), were used to construct galactose-inducible versions of the *DSK2* gene. The *DSK2* genes were isolated by digestion with BamHI and ligated into pMR613 at the BamHI site. The resulting plasmids, pMR2873 (*DSK2-1*) and pMR2872 (*DSK2+*), are 2-μm, *URA3* plasmids with *DSK2* under the control of the *GAL1* promoter (Yocum et al., 1984). Centromere-based plasmids were constructed by isolating the P_{GAL}-*DSK2* EcoRI/SalI 2-kb fragment from pMR2873 and pMR2872 and ligating it into pMR1868 cut with EcoRI/XhoI. This generated plasmids pMR2905 (P_{GAL}-*DSK2+*) and pMR2906 (P_{GAL}-*DSK2-1*). All experiments reported in this paper used the centromere-based versions of P_{GAL}-*DSK2*.

DSK2 Deletion and Integration Plasmids

To generate a two-step *DSK2* gene replacement plasmid, a series of plasmids were constructed. First, the *DSK2+* gene was removed from pMR2757 by digestion with XbaI and BamHI, leaving the 3′ flanking 540 bp of the *DSK2* gene on the plasmid. This was ligated with the *LEU2+* gene isolated as a 2-kb XbaI/BamHI fragment to generate pMR2819. This plasmid was then digested with BamHI, which was ligated with 430 bp of the 5′ flanking region of the *DSK2* gene (which was PCR amplified using primers 5′ GTC/GGA/TCC/GAT/CCG/TCT/TAT/TTG/CCT/CTC/G 3′ and 5′ CAA/GAA/TCC/CCG/TAG/C 3′). This generated pMR2831, a centromere-based *URA3* plasmid containing the *LEU2* gene flanked by 3′ and 5′ *DSK2* sequence. pMR2831 was digested with BglII/SpeI to remove the centromere, the ends were filled in with Klenow, and the vector was religated to obtain pMR2860, a vector that can be used for two-step gene replacement of *DSK2*.

To test linkage between the library clones and the *DSK2-1* suppressor, a 2.5-kb XhoI/HIII fragment that suppressed *kar1Δ17* was cloned into the *LEU2+* integrating vector pRS405 (Sikorski and Hieter, 1989) to generate pMR3161. The plasmid was linearized with NcoI to direct integration into a *kar1Δ17 DSK2+* strain (MS2373). The integration was confirmed by Southern analysis. Since the *kar1Δ17* allele has a tendency to diploidize (Vallen et al., 1992*a*), the strain MS2373 contained a *KAR1+*, *URA3* plasmid to ensure spore viability during a cross. After dissection and selection on 5-FOA, the spores were tested for temperature resistance to follow the suppression of the *kar1Δ17* allele. Six tetrads segregated 4:0 for temperature resistance, indicating that the suppressing activity present on the integrating DNA corresponded to the *DSK2-1* locus. To confirm this, the spores from one tetrad were backcrossed to a *kar1Δ17*, *DSK2+* strain (MS2372) and dissected. Temperature resistance of these spores segregated 2:2, indicating that the authentic *DSK2-1* locus had been cloned.

DSK2-1 Library Construction

One liter of strain MS3145 (*DSK2-1, kar1Δ17*) was grown to OD 600 of 1.7 in YPD and harvested to isolate genomic DNA as described in Gaber et al. (1990). To construct the library, Sau3A partial enzyme digestions were performed as described in Rose and Broach (1991) and subsequently fractionated on 10–40% sucrose gradients as described in Current Protocols in Molecular Biology (Ausubel et al., 1994). The 5–10-kb and 10–20-kb fractions from the gradients were pooled and ligated into vector pRS414 (Sikorski and Hieter, 1989) at the BamHI site as described in Rose and Broach (1991). To amplify the library, ligations were electroporated into strain XL1-Blue (Bullock et al., 1987) and plated on LB-amp plates. At least 35,000 colonies were pooled and DNA was isolated (Sambrook et al., 1989). Random screening of 20 plasmids indicated at least 60% of the plasmids contain inserts with an average size of 10 kb, representing a library of at least 14 genomes. The library reference numbers are pMR3173–3178.

DSK2 Cloning, Mapping, and Sequencing

The *DSK2-1* library was transformed into *kar1Δ17* (MS2082), and ~50,000 transformants (representing ~14 genomic equivalents) were grown at 23°C and subsequently replica printed to 37°C. 17 temperature-resistant colonies were isolated, retested, and checked for plasmid-linkage by selecting segregants that lost the plasmid by growth on 5-FOA. All 17 strains depended on the plasmid to be temperature resistant, so the plasmids were isolated from yeast by the method of Hoffman and Winston (1987), amplified in bacteria, and retransformed into *kar1Δ17* (MS2082). All 17 plasmids once again conferred temperature resistance upon *kar1Δ17*. Restriction mapping indicated the 17 plasmids represented 11 unique plasmids, pMR3162–3172, that contained common internal 1.3- and 4.2-kb RI/HIII fragments. The outer regions were mapped, and the smallest clone, pMR3165, contained ~7 kb as shown in Fig. 1 *A*.

To localize the *DSK2* gene, the subclones shown in Fig. 1 *A* were constructed, transformed into *kar1Δ17* (MS2082), and tested for temperature sensitivity. We found that the 4.2-kb RI/HIII subclone consistently suppressed *kar1Δ17* to levels comparable to the *DSK2-1* suppressor. Smaller subclones that were constructed (shown in Fig. 1 *A*) from this 4.2-kb plasmid still suppressed *kar1Δ17*, but resulted in reduced colony sizes at both 23° and 37°C. Therefore, the entire 4.2-kb EcoRI/HIII subclone (pMR3153) that suppressed *kar1Δ17* was sequenced. Decreased colony size observed when the 4.2-kb fragment was further subcloned may be due to toxicity of expressing the NH_2 terminus of the adjacent gene (Wolfe, D., and R. Keil, personal communication).

Both strands of the 4.2-kb *DSK2* clone were sequenced and can be accessed through EMBL/GenBank/DDBJ accession number L40587. The 1,852 bp that include the *DSK2+* gene are shown in this publication. We named the gene adjacent to *DSK2* the *DAG1* gene (*DSK2* adjacent gene), and later learned it corresponds to the *ZZZ1* gene that is involved in response to anesthesia (Wolfe, D., and R. Keil, personal communication). This gene is also entered in GenBank as the *BUL1* gene with accession number D50083. To facilitate *DSK2* sequencing, the 4.2-kb subclone was excised from pMR3165 using HIII and ligated into Bluescript vectors (Stratagene, La Jolla, CA) to allow exonuclease III digestions to be performed by the method of Clark and Henikoff (1994). The exonuclease deletions from clones pMR2833 and pMR2834 were then sequenced using T7 or T3 primers (Princeton University) complementary to the vector DNA with the Sequenase version 2.0 kit supplied by United States Biochemical Corp. (Cleveland, OH) according to double-strand sequencing method provided by the company. Gaps that remained were sequenced

with specific primers designed to hybridize to *DSK2*. Homology searches were performed using the sequence analysis software package by Genetics Computer Group, Inc. (Madison, WI).

To physically map *DSK2*, a 300-bp PstI fragment was labeled by the random primer method of Feinberg and Vogelstein (1983) and used to hybridize to lambda prime clone filters provided by Linda Riles (Washington University School of Medicine, St. Louis, MO) according to the provided protocol.

Since the *DSK2-1* mutant allele was cloned and sequenced first, the wild-type *DSK2+* gene and the *DSK2-2* suppressor were recovered by the gap-repair technique (Orr-Weaver et al., 1983). *DSK2* was removed from a centromere-based plasmid containing the *DSK2-1* gene by digestion with XbaI and McsI. The gapped plasmid was transformed into *kar1Δ17* strains containing either the wild-type *DSK2+* (MS2082) or *DSK2-2* (MS3134) alleles. DNA from the yeast transformants was isolated, and restriction analysis was performed to confirm that the gap was repaired. The gap-repaired plasmids were then transformed into *kar1Δ17* (MS2082) to test for suppression of the temperature sensitivity. Plasmids isolated from the wild-type *DSK2+* strain (MS2082) did not suppress the *kar1Δ17* defect, indicating that the wild-type *DSK2+* gene was recovered. Plasmids isolated from the *DSK2-2* strain (MS3134) suppressed *kar1Δ17*, indicating that the *DSK2-2* suppressor was recovered. The *DSK2* gene on these plasmids was sequenced, and we found a single base pair difference between the *DSK2-1* gene and the *DSK2+* gene: codon 69 is changed from a TAT in *DSK2-1* to a CAT in *DSK2+*. The *DSK2-2* suppressor contained the same mutation as the *DSK2-1* suppressor.

Immunological Techniques

Antibodies against bacterially expressed Dsk2p were generated in two female New Zealand White rabbits at the Princeton University Animal Facility. GST-Dsk2p protein (strain MR2865) was purified according to Smith and Johnson (1988) in the presence of protease inhibitors (chymostatin, leupeptin, aprotinin, pepstatin A, and AEBSF at 2 mg/ml final concentration and PMSF at 1 mM final concentration). AEBSF was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA), and all other protease inhibitors were from Sigma Chemical Co. (St. Louis, MO). The GST-Dsk2p was eluted from the glutathione beads by competition with 10 mM glutathione (Sigma Chemical Co.) for 60 min at room temperature. The thrombin-cleaved protein was separated on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH), and the band containing Dsk2p was excised. The nitrocellulose strip, which contained at least 0.5 mg of Dsk2p, was dissolved in DMSO. This mixture was emulsified with Freund's complete adjuvant (Sigma Chemical Co.) and injected subcutaneously. Boosts were performed monthly with at least 100 mg of this same mixture emulsified in Freund's incomplete adjuvant (Sigma Chemical Co.).

To purify full-length and NH_2-terminal Dsk2p antibodies, GST-Dsk2p and GST-ub-like domain recombinant protein were purified as described above, except the proteins were not thrombin cleaved. Approximately 0.5 mg of the soluble proteins were conjugated to Sulfo-link resin using the Sulfo-link kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's specifications.

Yeast extracts for Western blotting were prepared according to Ohashi et al. (1982). For Western blotting, proteins were separated on denaturing polyacrylamide gels, transferred to nitrocellulose (Schleicher & Schuell), and then Western blotted as described in Biggins and Rose (1994). Affinity purified anti-Dsk2p antibodies were used at a 1:1,000 dilution, affinity-purified anti-Cdc31p antibodies (Biggins and Rose, 1994) were used at a 1:500 dilution, and anti-ubiquitin antibodies (East Acres Biologicals, Southbridge, MA) were used at a 1:1,000 dilution. Secondary antibodies were obtained from Amersham Corp. (Arlington Heights, IL) and used at a 1:2,000 dilution followed by enhanced chemiluminescence according to manufacturer's directions. For competition experiments, various amounts of purified GST-Dsk2p ubiquitin-like domain recombinant protein were included during the primary incubations.

For the P_{GAL} shut-off experiments, strains MY3520 (pMR2903), MY3521 (pMR2904), MY3522 (pMR2905), MY3523 (pMR2906), and MY3516 (pMR613) were grown overnight at 30°C in SC-ura media with 2% raffinose to early logarithmic phase. Galactose was added to 2% final concentration for 2 h to induce the expression of the Dsk2 proteins. Glucose was then added to 2% final concentration to repress synthesis of the Dsk2 proteins. Equal volumes of cells were harvested at 0, 3, 7, and 9 h after glucose addition, and protein extracts were prepared and separated on a denaturing polyacrylamide gel. After transfer to nitrocellulose, a Western blot was performed to detect the levels of Dsk2p. As an internal loading control, the blots were also probed with anti-Kar2p antibodies (Rose et al., 1989).

Partially purified SPBs were prepared in collaboration with Dr. Mark Winey (University of Colorado, Boulder, CO) under the direction of Dr. Mike Rout (Rockefeller University, New York) according to the protocol of Rout and Kilmartin (1990). Briefly, spheroplasts were made from *Saccharomyces uvarum* and lysed. A crude nuclear pellet was loaded onto a gradient of layers 2.0, 2.1, and 2.3 M sucrose-polyvinylpyrolidone. The nuclei from the 2.1/2.3 fraction were collected and lysed, and SPBs were subsequently enriched on a step sucrose gradient. The steps in this gradient are: the sample layer, 1.75 M, 2 M, 2.25 M, and 2.5 M sucrose, all as described in Rout and Kilmartin (1990).

Pulse-chase Experiments

Pulse-chase experiments were performed essentially as described by Gammie et al. (1995) on 4.5 OD units of strains MS1554 and MS3759. The ^{35}S-translabel was added for a pulse of 5 min at 30°C, and 0.5-ml aliquots of cells were removed at 0, 2, 4, 8, and 24 h during the chase. The labeled cells were lysed with glass beads by vortexing in buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1% SDS), boiled for 4 min, and then spun for 10 min in a microfuge. The supernatant was diluted fourfold with buffer (60 mM Tris-Cl, pH 7.4, 6 mM EDTA, 190 mM NaCL, 1 mM PMSF, 0.1% Triton X-100) to a final SDS concentration of 0.2%. Two ml of anti-Dsk2p or anti-Cdc31p antibodies were added overnight at 4°C. Protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was added for 2 h at room temperature, and the beads were then washed twice each with high salt wash (1% Triton X-100, 0.2% SDS, 0.5 M NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.4), IP wash (1% Triton X-100, 0.2% SDS, 5 mM EDTA, and 0.15 M NaCl), and then detergent-free wash. SDS sample buffer (Biggins and Rose, 1994) was added to the beads, and the immunoprecipitates were separated on denaturing polyacrylamide gels, fixed in Amplify (Amersham Corp.) for 30 min, dried, and autoradiographed.

Microscopy

Indirect immunofluorescence using formaldehyde fixation of intact cells was performed as described by Rose et al. (1990), and indirect immunofluorescence using MeOH, acetone fixation of spheroplasts, was performed as described by Rout and Kilmartin (1990). Two different methods of fixation were used because the SPB antibodies do not stain formaldehyde-fixed cells, and because tubulin antibodies do not stain the methanol, acetone-fixed cells (Rout and Kilmartin, 1990; Biggins and Rose, 1994). Formaldehyde fixation was for 1 h at 23°C for all strains. Strains MY3516, MY3522, and MY3523 were grown to early logarithmic phase in SC-ura media containing 2% raffinose, and then galactose was added to 2% final concentration for 10 h and harvested for immunofluorescence. Strains MY3587, MY3588, MY3589, and MY3573 were grown to early logarithmic phase in YPD media at 30°C, shifted to 37°C for 6 h, and harvested. Strains MS10, MS3574, and MS3759 were grown to mid-logarithmic phase in YPD media and harvested. Affinity purified anti-Cdc31p antibodies (Biggins and Rose, 1994) were used at a 1:1,000 dilution, and affinity-purified anti-Dsk2p antibodies were used at a 1:100 dilution. Anti-90-kD antibodies and rat anti–tubulin antibodies were used at a 1:500 dilution and were the generous gift of John Kilmartin (Medical Research Council, Cambridge, UK). Rabbit anti–tubulin antibodies were also used at a dilution of 1:1,000 and were the generous gift of Frank Solomon (Massachusetts Institute of Technology, Cambridge, MA). Secondary antibodies were used at a 1:1,000 dilution (Boehringer Mannheim Biochemicals, Indianapolis, IN). Secondary antibodies for detection of Cdc31p and Dsk2p were FITC-conjugated goat anti–rabbit IgG. Anti-tubulin secondary antibodies were rhodamine-conjugated goat anti–rat IgG, and anti-90-kD secondary antibodies were rhodamine-conjugated goat anti-mouse IgG. 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Accurate Chemicals and Scientific Corp. (Westbury, NY).

EM was performed as described in Rose and Fink (1987). Strain MY3523 was grown to early logarithmic phase in SC-ura media containing 2% raffinose. Galactose was added to 2% final concentration for 6 h, and cells were harvested for EM. Strain MY4240 was grown to early logarithmic phase in YPD media, shifted to 37°C for 8 h, and harvested for microscopy.

Results

DSK2 Cloning and Mapping

We cloned the *DSK2-1* suppressor of the *kar1Δ17* mutant to elucidate its role in SPB duplication. Since the *DSK2-1* mutation is a dominant *kar1Δ17* suppressor with no other known phenotype, a centromere-based library was constructed from the *DSK2-1 kar1Δ17* strain (MS3145) to allow cloning by suppression of the *kar1Δ17* temperature sensitivity. The library was transformed into a *kar1Δ17* strain (MS2082) and grown at the permissive temperature of 23°C. To clone the *DSK2-1* gene, the colonies were replica printed to the nonpermissive temperature of 37°C and screened for temperature resistance.

Seventeen plasmids were identified that reproducibly conferred temperature resistance upon *kar1Δ17* (strain MS2082). Restriction analysis determined that the plasmids contained different overlapping regions of DNA from the same part of the genome. Plasmid integration and a linkage test confirmed that the cloned DNA corresponded to the authentic *DSK2* locus. To map the *DSK2* gene, a DNA restriction fragment common to all of the plasmids was used for hybridization to lambda-prime clone grid filters provided by Linda Riles. This determined that *DSK2* physically maps to the right arm of chromosome XIII near the *CDC65* gene.

To narrow down the minimal suppressing region of DNA, the smallest plasmid (~7.5 kb) was restriction mapped, and subclones were constructed (see Fig. 1 *A*) to test for suppression of *kar1Δ17*. Only the entire 4.2-kb subclone consistently suppressed *kar1Δ17* (Fig. 1 *A*) so this entire subclone was sequenced. Sequencing indicated that this subclone contained a complete open reading frame (designated ORF2 in Fig. 1 *B*) and a divergently transcribed partial open reading frame consisting of 776 amino acids (designated ORF1 in Fig. 1 *B*). Additional subclones mapped the suppressing activity to a 2-kb DNA fragment that contained the complete open reading frame (ORF2) and a small portion of the partial open reading frame. To determine which reading frame encodes the *DSK2* gene, the complete ORF2 was disrupted by inserting the *URA3+* gene into the NcoI restriction site (see Fig. 1 *B*). This plasmid could not suppress *kar1Δ17*, indicating the *DSK2* gene corresponds to the single complete open reading frame designated ORF2. In addition, this was further confirmed when the *DSK2-1* mutation was found to map within this open reading frame (see below). Partial suppression of the smaller subclones was likely to arise from toxicity of the NH_2-terminal part of ORF1 (Wolfe, D., and R. Keil, personal communication).

DSK2 Encodes a Ubiquitin-like Gene

The DNA sequence of the RI/HIII fragment is entered into GenBank under accession number L40587. The *DSK2* sequence is shown in Fig. 2 *A* and encodes an open reading frame of 373 amino acids that is predicted to encode a 41-kD protein. Homology searches indicated significant homology between the first 76 amino acids of Dsk2p and ubiquitin. Dsk2p is 36% identical and 55% similar to the yeast ubiquitin proteins (Ozkaynak et al., 1987; shown in Figs. 1 *C* and 2 *B*). Homology searches with the NH_2-terminal 76 amino acids of Dsk2p identified many ubiquitin homologues that have significant homology to Dsk2p. For example, Dsk2p is 52% similar and 31% identical to the first 76 amino acids of the yeast Rad23 protein (Watkins et al., 1993; see Figs. 1 *C* and 2 *B*) and 61% similar and 36% identical to the human XP-C proteins (Masutani et al., 1994; data not shown). Since all ubiquitin proteins are >90% identical and Dsk2p is 36% identical, we classify Dsk2p as a ubiquitin-like protein (see Fig. 1 *C*). Homology searches with the COOH-terminal domain of Dsk2p did not identify any proteins with significant homology, including the yeast Rad23 protein. Because we cloned the *DSK2-1* mutant allele first, the wild-type *DSK2+* gene and the *DSK2-2* suppressor were recovered by the gap-repair technique (Orr-Weaver et al., 1983) and sequenced. There is a single base pair change between

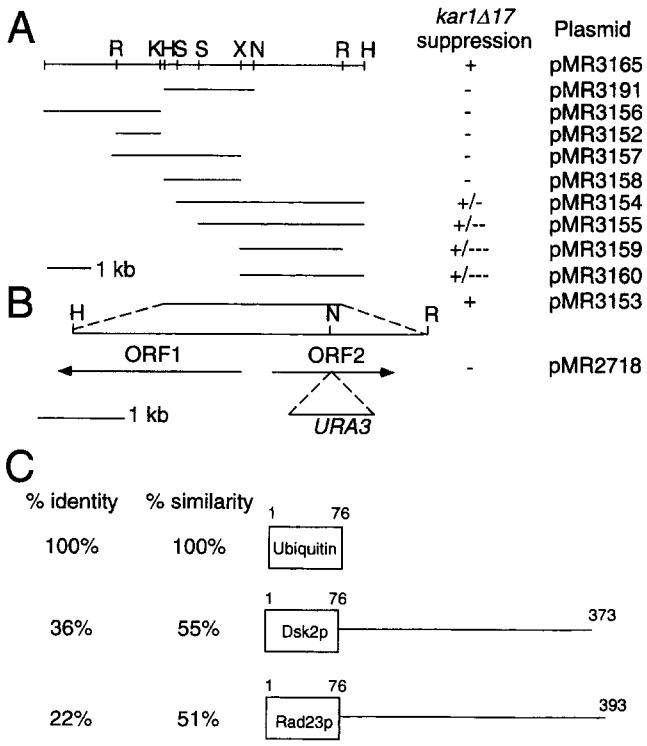


Figure 1. DSK2 Subclones. (*A*) pMR3165, the smallest *kar1Δ17* suppressing plasmid, was restriction mapped with a variety of enzymes (*R*, EcoRI; *K*, Kpn; *H*, HIII; *S*, SpeI; *X*, XhoI; *N*, NcoI) that are designated to scale. Smaller subclones were constructed as indicated and tested for *kar1Δ17* suppression at 37°C. The symbol (+) indicates suppression of *kar1Δ*17 to levels comparable to *DSK2-1*, while the symbol (−) indicates no suppression. Intermediate levels of suppression and papillation to wild type are indicated by the following (+/-, +/--, and +/---). (*B*) A 4.2-kb R/H subclone, pMR3153, suppressed *kar1Δ*17 to *DSK2-1* levels and was sequenced on both strands. This plasmid contained one partial open reading frame (ORF1) and a second complete open reading frame (ORF2). ORF2 was disrupted at the NcoI site with the *URA3* gene to generate pMR2718 as indicated. This plasmid did not suppress *kar1Δ17*, indicating that the *DSK2* gene corresponds to ORF2. (*C*) Structure of the Dsk2 protein. The NH_2-terminal 76 amino acids of Dsk2p and Rad23p (*boxes*) are 55% and 51% similar and 36% and 22% identical, respectively, to the ubiquitin protein (Ozkaynak et al., 1987; Watkins et al., 1993) and comprise ubiquitin-like domains. The ubiquitin-like proteins have unique COOH-terminal tail regions (*line*).

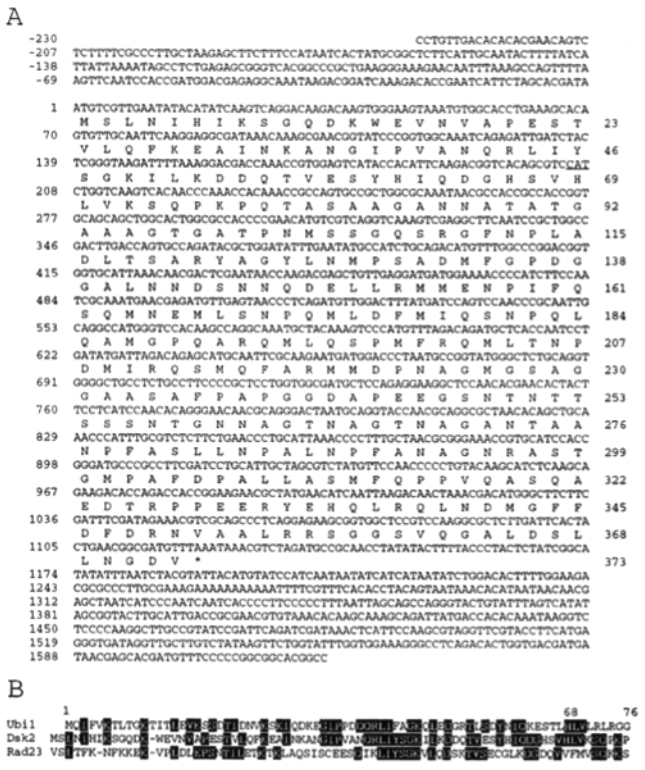


Figure 2. (*A*) *DSK2* sequence. The entire 4.2-kb plasmid pMR3153 (see Fig. 1) was sequenced and the 3′ 1,853 nucleotides (corresponding to ORF2 in Fig. 1 *B*) that include the *DSK2+* gene are shown. The *DSK2* open reading frame is designated to begin with nucleotide 1, and upstream nucleotides −230 to 1 are shown, as well as 518 downstream nucleotides. The *dsk2* deletion encompasses nucleotides +1 to +1130 to eliminate the entire *DSK2* gene. The nucleotide numbers are indicated at the left of the sequence. The *DSK2* gene encodes a 373–amino acid protein. The protein sequence is indicated under each codon with the amino acid numbers indicated on the right of the sequence. In the *DSK2-1* and *DSK2-2* mutants, codon 69 (*underlined*) is changed from CAT to TAT, which changes amino acid 69 from histidine to tyrosine. These sequence data are available from GenBank/EMBL/DDBJ under accession number L40587. (*B*) Dsk2p pileup. The first 76 amino acids of Dsk2p and Rad23p (Watkins et al., 1993), the ubiquitin-like domains, are aligned with the yeast ubiquitin protein (Ozkaynak et al., 1987). Identical residues are indicated by black boxes. The Dsk2p ubiquitin-like domain is 55% similar and 36% identical to ubiquitin and 52% similar and 31% identical to Rad23p. There is no significant homology between the COOH-terminal regions of Dsk2p and Rad23p. Amino acids numbers are indicated above the sequence.

DSK2-1 and *DSK2+*, which mutates amino acid 69 in the ubiquitin-like domain from a histidine in wild-type *DSK2+* to a tyrosine in *DSK2-1* (the codon is underlined in Fig. 2 *A*). Sequencing of the *DSK2-2* suppressor indicated it contains the same mutation as the *DSK2-1* suppressor, although the two alleles were isolated independently (Vallen et al., 1994). It seems likely that the *DSK2* suppressor is a very special form of the protein.

The Dsk2p Ubiquitin-like Domain Is Not Cleaved

The homology between Dsk2p and ubiquitin led us to test for similarities between the proteins. Full-length Dsk2p and Dsk2-1p were cloned into bacterial GST expression vectors to allow expression and purification of recombinant Dsk2 protein. When expressed in bacteria, the Dsk2 protein migrates at a molecular weight of ~41,000, consistent with the predicted molecular weight (data not shown). A Western blot was performed with anti-ubiquitin antibodies on the purified bacterial GST–Dsk2 fusion proteins from bacteria and determined that anti-ubiquitin antibodies weakly cross-reacted with the Dsk2 protein but not with GST protein (data not shown).

Since all ubiquitin proteins are synthesized as fusion proteins that are subsequently cleaved by cellular hydrolases to generate free ubiquitin, we tested whether the ubiquitin-like domain is cleaved from the Dsk2 protein. Ubiquitin has a glycine residue at amino acid 76 that is important for hydrolase recognition, and Dsk2p has a proline residue at this position, suggesting that Dsk2p would not be cleaved by the ubiquitin hydrolases. Nevertheless, a different set of hydrolases might exist to cleave Dsk2p. Polyclonal antibodies were generated against purified Dsk2 recombinant protein and subsequently purified on a bacterial Dsk2p affinity column. A Western blot on a total yeast protein extract using anti-Dsk2p antibodies determined that the Dsk2p antibodies recognize a single band in yeast corresponding to 41 kD (Fig. 3 *A*, lane *1*). This band is the same molecular weight as the bacterially expressed Dsk2p and is not present in a *dsk2Δ* yeast strain (Fig. 3 *A*, lane *2*). Since the Dsk2p antibodies recognize a single protein of the predicted full-length Dsk2 protein in yeast, the ubiquitin-like domain did not appear to be cleaved.

To rigorously determine whether the ubiquitin-like domain is cleaved, we purified antibodies specific to the NH_2-terminal ubiquitin-like (ub-like) domain. A GST fusion containing the Dsk2 ub-like domain was expressed and purified from bacteria and coupled to a column to purify antibodies specific to the Dsk2p ub-like domain. The NH_2-terminal antibodies and anti-Dsk2p full-length antibodies were used for Western blotting of total yeast extracts; the ub-like domain antibodies recognized the same band as the full-length antibodies (Fig. 3 *B*, lane *1*), indicating that the ub-like domain is present on the full-length protein. To confirm that the NH_2-terminal antibodies are specific to the ub-like domain, a competition experiment with pure recombinant Dsk2p GST-ub–like protein was performed. Western blotting of total yeast extracts with either Dsk2p NH_2-terminal antibodies or full-length antibodies was performed in the presence of increasing amounts of ub-like protein. The Dsk2p GST-ub–like protein fully competed away the Western blot signal of the anti-ub–like antibodies (Fig. 3 *C*), indicating that these antibodies are specific to the ub-like domain of Dsk2p. However, the ub-like protein could only partially compete away the signal of the full-length antibodies (Fig. 3 *C*), indicating that the full-length antibodies recognize the COOH-terminal domain of Dsk2p.

Dsk2p and Cdc31p Stabilities Are Not Altered by the DSK2-1 Mutation

One of the cellular functions of ubiquitin is to polyubiqui-

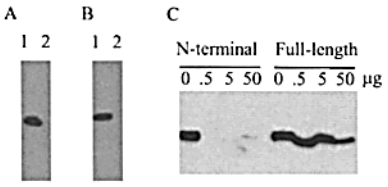


Figure 3. The Dsk2p ubiquitin-like domain is not cleaved. (*A* and *B*) Extracts from wild-type strains (MS1554, lane *1*) and *dsk2Δ* strains (MS3574, lane *2*) were prepared, separated on denaturing polyacrylamide gels, and transferred to membrane. (*A*) Affinity-purified full-length anti-Dsk2p antibodies used for Western blotting recognized a single band of 41 kD in wild-type extracts and no protein in *dsk2Δ* extracts. (*B*) Purified NH_2-terminal anti-Dsk2p antibodies used for Western blotting recognized the same band of 41 kD as the full-length antibodies. (*C*) A competition experiment was performed to show that the NH_2-terminal Dsk2p antibodies are specific to the NH_2 terminus and recognize the same size protein as the full-length antibodies. 0, 0.5, 5, or 50 mg of purified NH_2-terminal Dsk2 protein (indicated above the blots) was incubated with either NH_2-terminal Dsk2p antibodies or full-length antibodies during Western blotting of wild-type yeast strains. The Western blot shows that 0.5 mg of pure NH_2-terminus is sufficient to compete away the binding of the NH_2-terminal Dsk2p antibodies, but 50 mg of NH_2-terminal protein cannot fully compete away the binding of the full-length Dsk2p antibodies. Therefore, the NH_2-terminal antibodies are specific to the NH_2 terminus and recognize the same protein as the full-length antibodies, showing that the ubiquitin-like domain of Dsk2p is not cleaved.

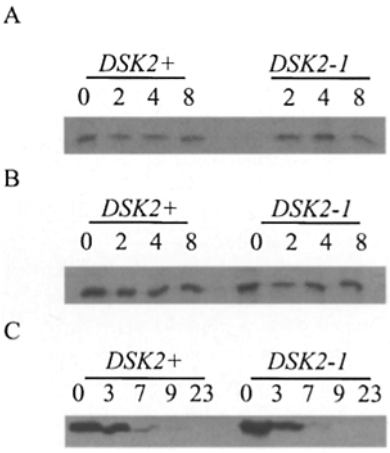


Figure 4. The stabilities of Dsk2p and Cdc31p are not altered in *DSK2-1* strains. (*A* and *B*) A pulse-chase experiment was performed on *DSK2+* and *DSK2-1* strains to determine the stability of Cdc31p and Dsk2p. Equivalent volumes of labeled cells were harvested at 0, 2, 4, and 8 h (indicated above the blots), and proteins were immunoprecipitated with anti-Cdc31p (*A*) or anti-Dsk2p (*B*) antibodies. The proteins were resolved on denaturing polyacrylamide gels and subjected to autoradiography that indicated no differences in stability between the strains. (*C*) A P_{GAL}-*DSK2* shut-off experiment. The *DSK2+* and *DSK2-1* genes were placed under the control of the galactose-inducible *GAL1* promoter and expressed in *dsk2Δ* strains as the sole source of Dsk2 protein. Protein synthesis was repressed by the addition of glucose to the media, and equivalent numbers of cells were harvested at 0, 3, 7, 9, and 23 h (indicated above the blots) after the addition of glucose. Extracts from the cells were prepared, separated on a denaturing polyacrylamide gel, and transferred to a membrane. Western blots of the membrane with anti-Dsk2p antibodies are shown and indicate that the stability of the Dsk2p and Dsk2-1 proteins is equivalent.

tinate substrates on one or more lysine residues to cause substrate degradation via the proteasome (for review see Ciechanover, 1994). Although the details of this process are not completely understood, site-directed mutagenesis on ubiquitin was performed to define residues that are important for the resulting degradation of substrates (Ecker et al., 1987). In this study, it was determined that one of the ubiquitin residues important in the subsequent degradation of substrates is histidine-68. When this residue was mutated to a lysine, the BSA substrate that was conjugated to the mutant ubiquitin was only degraded at 30% the rate of wild-type ubiquitin-conjugated BSA. Although the mechanism of the change in stability is unknown, the mutation in ubiquitin somehow decreases substrate proteolysis. Since both the *DSK2-1* and *DSK2-2* mutations change histidine-69 (which corresponds to the ubiquitin histidine 68) to a tyrosine, it was possible that they affect the stability of a ubiquitinated protein by inhibiting proteolysis.

We therefore tested the stability of Dsk2p and Cdc31p in *DSK2+* and *DSK2-1* strains since mutations in both genes suppress the *kar1Δ17* temperature sensitivity. Pulse-chase experiments were performed on wild-type (MS1554) and *DSK2-1* (MS374) strains by adding ^{35}S-translabel for 5 min and harvesting cells at different times during the chase. The cells were lysed, and immunoprecipitations with anti-Dsk2p and anti-Cdc31p were performed, separated on denaturing gels, and subjected to autoradiography to determine the amount of labeled protein. This determined that both Cdc31p (Fig. 4 *A*) and Dsk2p (Fig. 4 *B*) have very long half-lives (>8 h) in wild-type and *DSK2-1* strains, so a change in their stability is unlikely to be the mechanism of *DSK2-1* suppression of *kar1Δ17*. We also tested the stability of Cdc31p and Dsk2p in a *kar1Δ17* strain at the permissive and nonpermissive temperatures by pulse-chase to determine whether their stability was altered during suppression. Again, the half-lives of Cdc31p and Dsk2p were very long and therefore unlikely to be relevant to the suppression (data not shown). In addition, pulse-chase experiments with very short and long time points were conducted, and we did not detect the presence of any alternative forms of Dsk2p, supporting our observation that the NH_2-terminal domain of Dsk2p is not cleaved (see above).

To determine by an alternative method whether Dsk2p and Dsk2-1p have equal half-lives, a galactose shut-off experiment was performed. The *DSK2+* and *DSK2-1* genes were placed under the control of the galactose inducible *GAL1* promoter (Yocum et al., 1984). They were transformed into a *dsk2Δ* strain (MS3574, see below) and grown in galactose for 2 h to induce expression of the Dsk2 proteins. Glucose was added to repress further synthesis of the proteins, and aliquots of cells were harvested at various time points. When equal numbers of cells were subjected to a Western blot with anti-Dsk2p antibodies to monitor the protein levels, the turnover rate of Dsk2p and Dsk2-1p appeared to be equivalent (Fig. 4 *C*).

DSK2-1 Requires the COOH-terminal Tail for Function

Since the *DSK2-1* mutation maps to the ubiquitin-like domain, we tested whether the ub-like domain is sufficient for the suppression of *kar1Δ17*. The wild-type and *DSK2-1* ub-like domains were cloned into a centromere-based yeast vector under the control of the regulated galactose promoter. They were then transformed into wild-type (MS1554) and *kar1Δ17* (MS2082) strains. To check that the ub-like proteins are expressed and stable, the strains were grown in the presence of galactose, and yeast extracts from these strains were prepared and separated on denaturing gels. The proteins were transferred to membranes and probed with anti-Dsk2p antibodies to detect the protein. The proteins were expressed to the same level as the full-length Dsk2p (data not shown). These strains were then grown at 37°C to test for suppression of *kar1Δ17*. The ub-like domain of *DSK2+* and *DSK2-1* did not suppress *kar1Δ17*, indicating that the tail of the protein is important for the suppression (data not shown). In addition, the overexpression of the truncated proteins was not toxic, unlike overexpression of the full-length protein (see below).

DSK2 and RAD23 Are Involved in SPB Duplication

To learn more about the function of the *DSK2* gene, a two-step gene replacement method (Orr-Weaver et al., 1983) was used to delete the entire *DSK2* gene. The deletion did not affect growth rate at temperatures ranging from 23° to 37°C. Since the *dsk2Δ* had no detectable phenotype, it seemed possible this might be due to functional redundancy with another gene(s) such as the ubiquitin-like gene, *RAD23*. Although a *rad23Δ* strain is UV sensitive (Watkins et al., 1993), it displays no growth defects at temperatures ranging from 23° to 37°C. To test for functional redundancy between the ubiquitin-like genes, we generated a *rad23Δ dsk2Δ* double mutant. The growth rate of the double mutant was normal at 23° and 33°C (Table II), but growth was severely inhibited at 35° and 37°C (Table II). Growth curves were performed on wild-type, *dsk2Δ*, *rad23Δ*, and *dsk2Δ rad23Δ* strains at 37°C. The *dsk2Δ*, *rad23Δ*, and wild-type strains had similar growth rates (Fig. 5 *A*). Growth of the *dsk2Δ rad23Δ* double mutant arrested within 1 h after the shift to the nonpermissive temperature (Fig. 5 *A*).

To determine whether the temperature-sensitive defect in the *dsk2Δ rad23Δ* double mutant was due to a defect in SPB function, indirect immunofluorescence was performed to visualize the microtubules, the SPBs, and Cdc31p localization. Wild-type, *dsk2Δ*, *rad23Δ*, and *dsk2Δ rad23Δ* strains were grown to early logarithmic phase at 30°C, shifted to 37°C for 6 h, and formaldehyde fixed for indirect immunofluorescence. The wild-type and single mutant strains had normal distributions of cells in each phase of the cell cycle (Fig. 5 *C*). In the *rad23Δ dsk2Δ* strain, there was an increase in large-budded cells to 47% (compared to 11% in the isogenic wild-type strain; Fig. 5 *C*). DAPI staining to visualize the nucleus in these cells showed that the nucleus was abnormally positioned in the neck of the large-budded cells, resulting in a "cut" phenotype (Fig. 6 *K*). In contrast, the wild-type, *rad23Δ*, and *dsk2Δ* strains had either an elongated nucleus or two nuclei in large-budded cells (Fig. 6, *B*, *E*, and *H*). The *rad23Δ* strain had a small percentage (5%) of large-budded cells with a single nucleus positioned in the neck (Fig. 5 *C*). Immunofluorescent staining of tubulin to visualize the microtubules determined that the wild-type, *dsk2Δ*, and *rad23Δ* strains had wild-type cytoplasmic and nuclear microtubule morphology (Fig. 6, *C*, *F*, and *I*). In large-budded cells, the microtubules in these strains extended between the two nuclei, indicating the presence of an elongated bipolar mitotic spindle (Fig. 6, *C*, *F*, and *I*). The small percentage of large-budded cells with the cut phenotype in the *rad23Δ* strain contained a short bipolar spindle that extended between the two edges of the nucleus as defined by the DAPI staining (data not shown). In contrast, the large-budded cells in the *dsk2Δ rad23Δ* strain had a single vertex of microtubules on the edge of the nucleus, indicating the presence of a monopolar spindle (Fig. 6, *L*). This defect suggested that the large-budded cells in the *dsk2Δ rad23Δ* strain had either a single SPB or duplicated SPBs that had not separated.

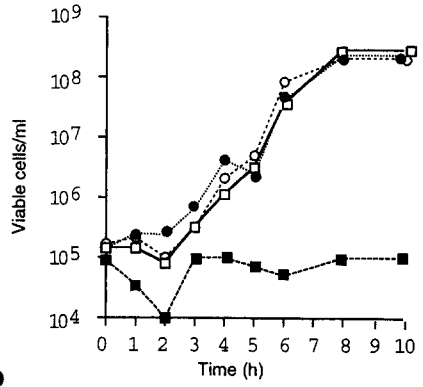
To visualize the SPBs, indirect immunofluorescence was performed using anti-90-kD antibodies generated against partially purified SPBs by Rout and Kilmartin (1990) or anti-Cdc31p antibodies (Biggins and Rose, 1994). Staining with SPB antibodies on wild-type, *dsk2Δ*, and *rad23Δ* strains was normal: the distribution of cells with one and two SPBs matched expectations based on the distribution of cells in the cell cycle (Fig. 5 *C*). Unbudded cells and a few small-budded cells should have a single SPB, and all other cells should have two SPBs. In wild-type, *dsk2Δ*, and *rad23Δ* strains, ~60% of the cells had a single SPB as visualized as a dot of 90-kD staining (Fig. 7, *B*, *D*, and *F*) on the edge of the nucleus as defined by DAPI staining (Fig. 7, *A*, *C*, and *E*). This percentage correlates well with the ~60% unbudded cells (Fig. 5 *C*). In contrast, there was an increase in the percentage of cells containing a single SPB from 60% to 87% in the *dsk2Δ rad23Δ* strain at 37°C (Figs. 5 *C* and 7 *H*). We attribute the increase to 87% of single SPBs to the increase in large-budded cells with a monopo-

Table II. Genetic Interactions between CDC31, KAR1, DSK2, and RAD23

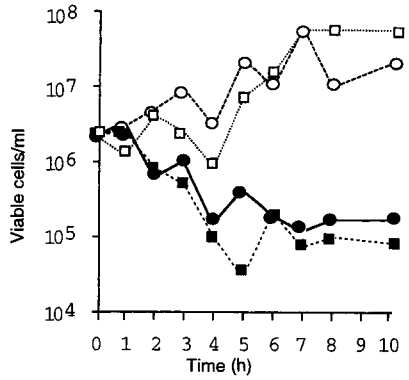
Strain	Plasmid	23°C	33°C	35°C	37°C
kar1Δ17	vector	+	+	−	−
	2μ-*CDC31*$^+$	+	+	+	+/−
	CDC31-16	+	+	+	−/+
	DSK2-1	+	+	+	−/+
dsk2Δ rad23Δ	vector	+	+	−	−
	2μ-*CDC31*$^+$	+	+	+	−
	CDC31-16	+	+	+	−
	DSK2-1	+	+	+	+
kar1Δ17 dsk2Δ rad23Δ	vector	+	−	−	−
	2μ-*CDC31*$^+$	+	+	−/+	−
	CDC31-16	+	+	+	−
	DSK2-1	+	+	+	−/+
Wild type	vector	+	+	+	+
	2μ-*CDC31*$^+$	+	+	+	+
	CDC31-16	+	+	+	+
	DSK2-1	+	+	+	+

Strains were transformed with plasmids as indicated, and growth was tested at the various temperatures.

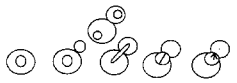
A



B



C



						1 SPB	2 SPB
WILDTYPE	66	22	11	0	<1	60	40
dsk2Δ	64	26	11	0	<1	61	39
rad23Δ	56	25	13	5	0	60	40
dsk2Δ rad23Δ	48	5	8	0	39	87	13
pGAL-VECTOR	67	21	11	0	0	62	38
pGAL-*DSK2+*	57	18	4	20	1	53	47
pGAL-*DSK2-1*	42	15	8	0	35	68	32

Figure 5. *dsk2Δ rad23Δ* strains and *DSK2* overexpression strains have defects in SPB duplication. (*A*) A growth curve was performed on wild-type (*open squares*), *dsk2Δ* (*closed circles*), *rad23Δ* (*open circles*), and *dsk2Δ rad23Δ* (*closed squares*) strains at 37°C. Cells were plated every hour, and the number of viable cells were calculated. The *dsk2Δ rad23Δ* strain arrests after 1 h, while the other strains have equivalent growth rates at 37°C. (*B*) A growth curve was performed on P_{GAL}-*DSK2+* (*circles*) and P_{GAL}-*DSK2-1* (*squares*) strains that were grown in glucose (*open*) or galactose (*closed*) for 10 h at 30°C. Cells were plated every hour, and the number of viable cells was calculated. The overexpression of both genes causes decreased viability. (*C*) The distribution of cells in wild-type, *dsk2Δ*, *rad23Δ*, and *dsk2Δ rad23Δ* strains after 6 h at 37°C were calculated by counting cells that were formaldehyde fixed. Greater than 200 cells were counted for each strain, and the percentage of each type of cell is indicated below the pictorial representations. The pictures above each column represent the following, respectively: unbudded cells, small-budded cells, large-budded cells with nuclei that have separated or are in the process of separating, large-budded cells with a single nucleus in the neck and a short spindle, and large-budded cells with a monopolar spindle. In addition, the percentage of cells containing one or two SPBs was calculated by counting >200 cells that were stained with anti-SPB antibodies (90-kD or Cdc31p). The experiment was performed twice, and the averages of both experiments are reported. The same experiment was performed on cells containing the *DSK2+* and *DSK2-1* genes under control of the *GAL1* promoter. The genes were overexpressed by growth in galactose for 10 h and processed as described above.

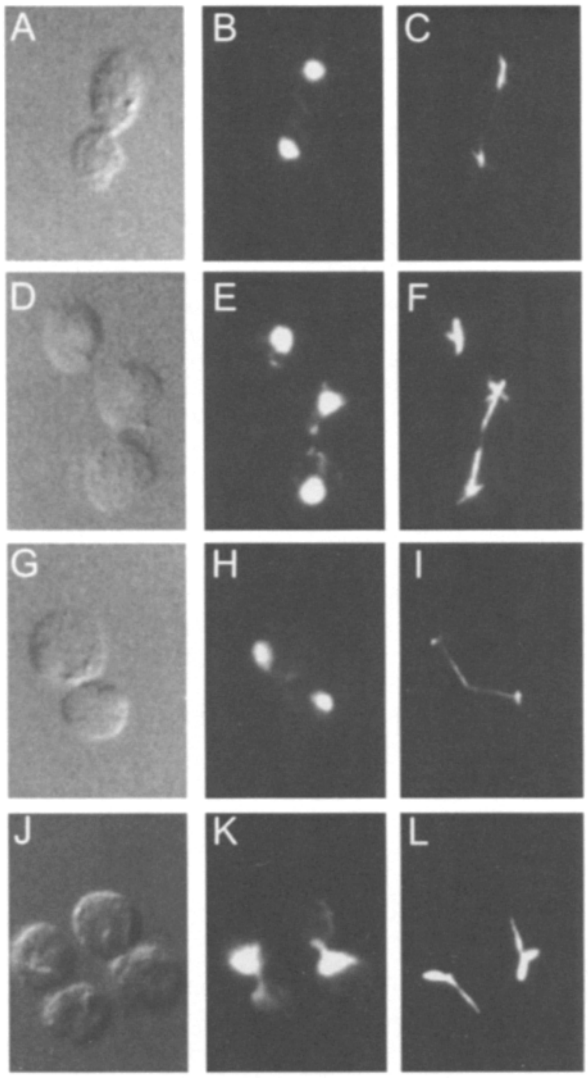


Figure 6. Indirect immunofluorescence on wild-type, *dsk2Δ*, *rad23Δ*, and *dsk2Δ rad23Δ* strains. Wild-type (*A–C*), *rad23Δ* (*D–F*), *dsk2Δ* (*G–I*), and *dsk2Δ rad23Δ* (*J–L*) strains were grown at 37°C for 6 h and fixed for indirect immunofluorescence. Nomarski optics are shown in *A*, *D*, *G*, and *J*, DAPI staining in *B*, *E*, *H*, and *K*, and anti-tubulin staining in *C*, *F*, *I*, and *L*. Large-budded cells in all strains except the *dsk2Δ rad23Δ* strain have wild-type tubulin staining indicated by an elongated mitotic spindle (*C*, *F*, and *I*) with separated DNA (indicated by DAPI staining in *B*, *E*, and *H*). In the *dsk2Δ rad23Δ* strain, the nucleus is stuck in the neck (indicated by DAPI staining in *K*), and there is a monopolar spindle indicated by the tubulin staining (*L*).

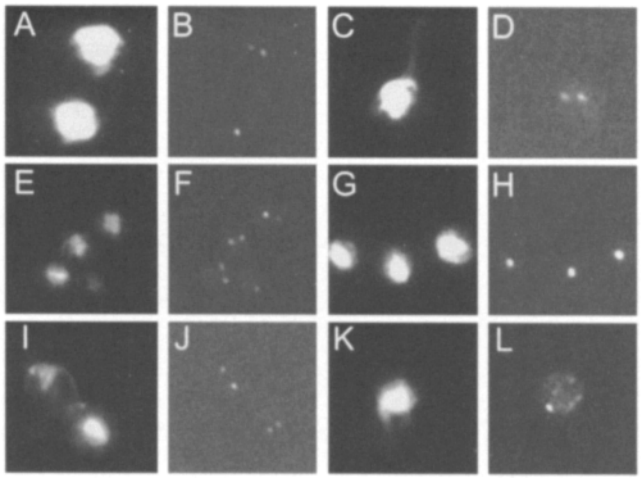


Figure 7. SPB staining of *dsk2Δ rad23Δ* strains and *DSK2* overexpression strains. To determine the frequency of cells containing one or two SPBs, anti-Cdc31p and anti-90-kD indirect immunofluorescent staining was performed on wild-type (*A* and *B*), *rad23Δ* (*C* and *D*), *dsk2Δ* (*E* and *F*), and *dsk2Δ rad23Δ* strains (*G* and *H*) grown at 37°C for 6 h. The frequencies of cells containing one or two SPBs are reported in Fig. 5 *C*. Anti-90-kD staining shown in *B*, *D*, *F*, and *H* indicates the SPB on the nucleus that is defined by DAPI staining in *A*, *C*, *E*, and *G*. The wild-type, *dsk2Δ*, and *rad23Δ* strains contain numerous cells containing two SPBs (*B*, *D*, and *F*). The *dsk2Δ rad23Δ* strain consists mainly of cells with a single dot of 90-kD staining and therefore contains many large-budded cells with a single SPB (*H*). The same experiment was performed on P_{GAL}-*DSK2* strains that were grown in galactose for 10 h. A strain containing the vector is shown in *I* and *J*, and a strain containing P_{GAL}-*DSK2-1* is shown in *K* and *L*. Anti-90-kD staining and anti-Cdc31p staining were the same in all strains, and anti-90-kD staining is shown in *J*, anti-Cdc31p staining in *L*, and DAPI in *I* and *K*. The overexpression of *DSK2-1* also causes the accumulation of large-budded cells containing a single SPB in which Cdc31p is still localized to the SPB (*L*).

lar spindle observed by tubulin staining. Few cells were ever seen in the *dsk2Δ rad23Δ* strain with adjacent SPBs, suggesting the monopolar tubulin staining is due to cells with unduplicated SPBs.

To determine whether the monopolar spindles in the *dsk2Δ rad23Δ* strain contain a single SPB or duplicated but unseparated SPBs, EM was performed. Serial sections of 11 large-budded cells with a single nucleus were analyzed. Each cell was followed through six to twelve sections. Seven of these cells (66%) contained a single SPB (Fig. 8, *A* and *B*). Absence of a second SPB in the preceding and subsequent sections confirmed that the SPB had not duplicated. The unduplicated SPBs were often enlarged and embedded within a deep invagination of the nucleus (Fig. 8, *A* and *B*), resembling the phenotype of *kar1* (Rose and Fink, 1987) and *cdc31* (Byers, 1981) mutants. The four remaining cells contained duplicated SPBs, either in a bipolar configuration (three of the four cells) or in a side by side configuration (one of the four cells) (data not shown). These results are in close agreement with the immunofluorescence data in which ~60% of the large-budded cells contained a monopolar spindle (Fig. 5 *C*). Therefore, the monopolar cell cycle block of the *dsk2Δ rad23Δ* strain was due to a block in SPB duplication.

Since the temperature-sensitive defect of *kar1Δ17* results in a SPB duplication defect due to mislocalization of Cdc31p, we tested whether Cdc31p was properly localized to the SPB in the *rad23Δ dsk2Δ* strain at 37°C. In all four strains, Cdc31p staining always colocalized with the 90-kD staining, indicating that Cdc31p localization is not altered in the double mutant (data not shown).

We next tested whether the *CDC31* mutant suppressors of *kar1Δ17* suppressed the temperature sensitivity of the *dsk2Δ rad23Δ* strain. This would indicate that the defect involved *CDC31* function. We found that both *CDC31-16* and high copy *CDC31+* suppressed the *rad23Δ dsk2Δ* temperature-sensitive strain at 35°C but not at 37°C (Table II). In addition, we isolated *CDC31+* multiple times in a screen for high copy suppressors of the temperature-sensitive defect of the *rad23Δ dsk2Δ* double deletion strain (Ivanovska, I., and M. Rose, unpublished results). Therefore, the *dsk2Δ rad23Δ* defect is at least partly mediated through Cdc31p.

Since *RAD23* is functionally redundant with *DSK2* for SPB duplication, we tested whether *DSK2* has a role in DNA repair like *RAD23*. To do this, wild-type, *rad23Δ*, *dsk2Δ*, and *rad23Δ dsk2Δ* strains were plated and subjected to increasing UV light. We found that the *rad23Δ* and *rad23Δ dsk2Δ* strains were both equally UV sensitive, and the *dsk2Δ* strain was not UV sensitive (data not shown).

DSK2 Overexpression Blocks SPB Duplication

The overexpression of the Kar1p SPB component is toxic and causes a large-budded arrest with a monopolar spindle (Rose and Fink, 1987). Indirect immunofluorescence on overexpressed Kar1p indicates it forms a large aggregate that is no longer localized to the SPB (Rose and Fink, 1987). Cdc31p is associated with this aggregate (Biggins and Rose, 1994), suggesting that the block in SPB duplication by overexpression of *KAR1* may be due to a titration of SPB components away from the SPB. To determine if *DSK2* overexpression has phenotypes similar to *KAR1* overexpression, we constructed plasmids in which both *DSK2+* and *DSK2-1* were placed under the control of the *GAL1* promoter (Yocum et al., 1984). When high levels of Dsk2p were induced by growth on galactose plates, growth was inhibited. To carefully assess the growth defects of overexpression of *DSK2+* and *DSK2-1*, growth curves were performed in the presence and absence of galactose. Cells from both strains were grown to early logarithmic phase in the presence of raffinose. Glucose was added to half of the culture, galactose was added to the other half, and cells were plated for viability on glucose plates every hour for 10 h. The cessation of growth indicated that overexpression of both *DSK2+* and *DSK2-1* was toxic (Fig. 5 *B*).

To determine if the toxicity of *DSK2* overexpression was due to a defect in SPB duplication, cells were grown in galactose or glucose for 10 h and processed for indirect immunofluorescence as described above. In the overexpressed *DSK2* strains (Fig. 5 *C*), there was an increase in the percentage of large-budded cells. Wild-type cultures had 11% large-budded cells while P_{GAL}-*DSK2+* had 25% and P_{GAL}-*DSK2-1* had 43% large-budded cells (Fig. 5 *C*). DAPI staining of the overexpressed *DSK2* strains indi-

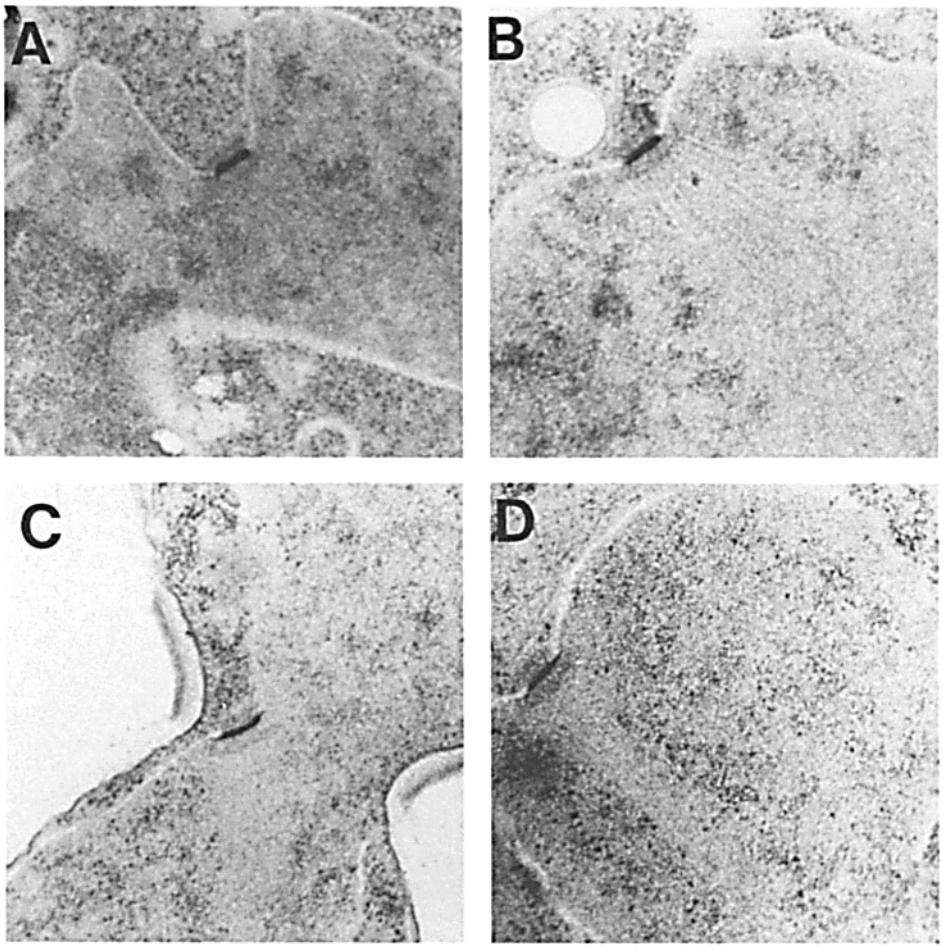


Figure 8. EM of a *dsk2Δ rad23Δ* strain and *DSK2-1* overexpression strain. A *dsk2Δ* rad23Δ strain (*A* and *B*) was grown at 37°C for 8 h and a P_{GAL}-*DSK2-1* strain (*C* and *D*) was grown in galactose for 6 h. Cells from both cultures were prepared for EM. Large-budded cells were chosen for analysis, and serial sections surrounding the SPB were examined. In 7 out of 11 large-budded *dsk2Δ* rad23Δ cells, a single SPB was observed spanning two to four serial sections. No additonal SPBs were observed in either neighboring sections or at the opposite end of the nuclear microtubule bundle. Two examples are shown in *A* and *B*. In three cells, duplicated SPBs were observed on the opposite side of the nucleus, at the other end of the nuclear microtubule bundle, indicating that a bipolar spindle had formed. In one cell, a duplicated SPB was found adjacent to the first SPB. These results indicated that the monopolar large-budded *dsk2Δ rad23Δ* cells were blocked in SPB duplication and not in SPB separation. The SPBs in serial sections of 12 large-budded cells overexpressing *DSK2-1* were also scored by the above criteria. Nine large-budded cells contained a single SPB (two examples are shown in *C* and *D*), and three contained a bipolar spindle. These results indicate that overexpression of Dsk2-1p also caused a block in SPB duplication and not in SPB separation.

cated that nuclei in the large-budded cells had an abnormal nuclear position in the neck of the cell (Fig. 9, *E* and *H*). Immunofluorescent staining of tubulin of these cells indicated that overexpression of *DSK2+* blocked cells with a short bipolar spindle (Fig. 9, *E* and *F*). Immunofluorescent staining of the anti-90 kD protein indicated that the percentage of cells with one SPB was lower than wild-type, and there was an increase in cells with two SPBs (Fig. 5 *C*). Therefore, the overexpression of *DSK2+* causes the accumulation of cells with short spindles. In contrast, anti-tubulin staining of the overexpressed *DSK2-1* large-budded cells indicated that they contained a monopolar spindle (Fig. 9 *I*) as defined by the single vertex of tubulin staining on the edge of the nucleus (Fig. 9 *H*). When immunofluorescent staining of the anti-90-kD protein was performed on these cells, we found an increased percentage of cells with a single SPB compared to wild-type cells (Fig. 5 *C*).

To confirm that the single SPB observed by immunofluorescence was due to a block in SPB duplication, serial section EM was performed on cells overexpressing *DSK2-1*. Serial sections of twelve large-budded cells that contained a single nucleus were followed through six to fifteen sections. Nine of the cells (75%) contained a single SPB (Fig. 8, *C* and *D*). The three other cells contained duplicated SPBs that were separated to opposite poles of the nucleus. The percentage of cells with a single SPB determined by EM (75%) correlated well with the percentage of cells containing a monopolar spindle determined by immunofluorescence (69%). Therefore, overexpression of *DSK2-1* also caused a block in SPB duplication.

To determine whether the SPB duplication defect could be due to Cdc31p mislocalization, immunofluorescent staining of anti-Cdc31p was performed on these strains. In all cases, anti-Cdc31p staining corresponded to the 90-kD staining (data not shown, but e.g., see Fig. 7 *L*), indicating that Cdc31p localization at the sole SPB was not affected.

DSK2, CDC31, and KAR1 Interactions

We previously determined that Kar1p and Cdc31p physically interact, and that the function of Kar1p in SPB duplication is to localize Cdc31p to the SPB (Biggins and Rose, 1994). To determine the relationship of the *DSK2* gene to

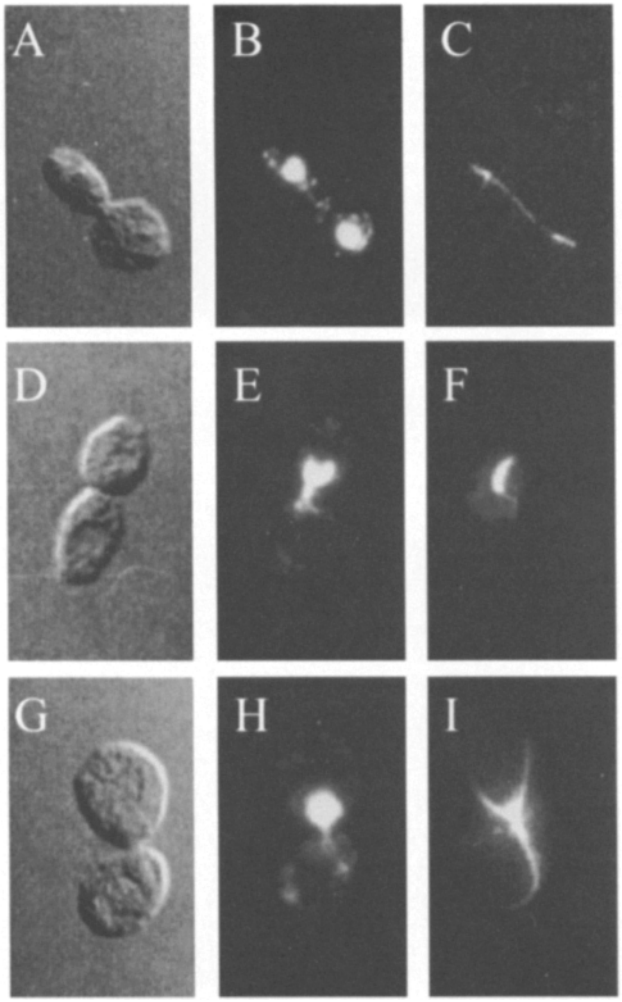


Figure 9. Indirect immunofluorescence on *DSK2* overexpression strains. Cells containing the P_{GAL} vector (*A–C*), P_{GAL}-*DSK2+* (*D–F*), and P_{GAL}-*DSK2-1* (*G–I*) were grown in galactose for 10 h and formaldehyde fixed for indirect immunofluorescence. Examples of large-budded cells indicated by Nomarski optics (*A*, *D*, and *G*) show that the nucleus in *DSK2* overexpressed strains (indicated by DAPI in *B*, *E*, and *H*) is abnormally positioned in the neck of the cell. In contrast to wild-type cells, which contain an elongated mitotic spindle as indicated by anti-tubulin staining (*C*), the P_{GAL}-*DSK2+* cells contain a short bipolar spindle (*F*), while P_{GAL}-*DSK2-1* cells contain a monopolar spindle (*I*).

the *KAR1* and *CDC31* genes, we tested genetic interactions between these genes. First, we tested for genetic interactions between *dsk2Δ* and *kar1Δ17* since *DSK2* is not an essential gene. A *dsk2Δ kar1Δ17* strain was generated and grew normally at 23° and 30°C. When the strain was tested for growth at 37°C, it was still temperature sensitive due to the *kar1Δ17* allele, indicating that there is no genetic interaction between the *dsk2Δ* and *kar1Δ17* at any temperature (data not shown). Since we found that the *DSK2* and *RAD23* genes are functionally redundant, we tested whether the *RAD23* gene interacted with *kar1Δ17*. A *rad23Δ kar1Δ17* strain was generated, and like the *kar1Δ17 dsk2Δ* strain, the *rad23Δ* did not display any genetic interactions with *kar1Δ17* (data not shown). We predicted that a *kar1Δ17 dsk2Δ rad23Δ* strain would exhibit a synthetic effect if the *KAR1*, *DSK2*, and *RAD23* genes genetically interact to initiate SPB duplication. We therefore generated a *kar1Δ17 rad23Δ dsk2Δ* strain and found it is temperature sensitive at 37°C (Table II), indicating that the *dsk2Δ rad23Δ* double deletion and *kar1Δ17* do not suppress each other. Strikingly, however, the triple deletion strain is now temperature sensitive for growth at 33°C (Table II), a temperature permissive for the *kar1Δ17* (Table II) and the *dsk2Δ rad23Δ* strains (Table II). This synthetic phenotype suggests that Dsk2p/Rad23p affect cell function in a manner related to Kar1p function.

We previously proposed that the *CDC31* alleles may suppress *kar1* mutants via an increased interaction with another SPB component since *CDC31* suppressor mutations restore the localization of Cdc31p to the SPB in a *kar1* deletion strain (Vallen et al., 1994; Biggins and Rose, 1994). We therefore tested whether the *DSK2* mutations also restore Cdc31p localization to the SPB in a *kar1* deletion strain. Indirect immunofluorescence was performed on a *kar1Δ2 DSK2-1* strain (MS2645) to localize Cdc31p. As shown in Fig. 10, Cdc31p staining (*B*) corresponds to the 90-kD staining (*C*) in the *kar1Δ DSK2-1* strain, showing that *DSK2-1* relocalizes Cdc31p to the SPB in the absence of *kar1*. If Dsk2p is the component that *CDC31* suppressors interact with to bypass *KAR1* function, then *DSK2* should be required for *CDC31-16* to suppress *kar1Δ17*. However, we constructed a *CDC31-16 dsk2Δ kar1Δ17* strain and found that *CDC31-16* suppressed *kar1Δ17* in either the presence or absence of *DSK2+* (data not shown). This indicates that Cdc31-16p suppression of *kar1Δ17* occurs either by interacting with factors in addition to Dsk2p or independent of Dsk2p. One potential candidate for this factor is the *RAD23* gene that is functionally redundant with *DSK2*. We therefore tested whether the *CDC31* suppressors can suppress the temperature sensitivity in a *kar1Δ17 dsk2Δ rad23Δ* strain. The *CDC31-16* allele (Table II) and high copy *CDC31+* (Table II) both suppress the triple deletion strain at 33°C and 35°C. As was found for the *dsk2Δ rad23Δ* strain, *CDC31* suppressors did not suppress the *dsk2Δ rad23Δ kar1Δ17* strain at 37°C (Table II). These data show that *RAD23* and *DSK2* are not required for *CDC31* suppression of *kar1Δ17*, nor is *KAR1* required for suppression of *rad23Δ dsk2Δ*. Taken together, these results indicate that *KAR1*, *RAD23*, and *DSK2* functions are mediated through the *CDC31* gene.

We next tested the genetic interactions between the *DSK2-1* and *CDC31-16* suppressors of *kar1Δ17*. Although *CDC31-16* is a dominant suppressor of the temperature-sensitive *kar1Δ17* mutation (Vallen et al., 1994), it is a recessive temperature-sensitive mutation in a wild-type *KAR1+* background. In addition, *CDC31-16* causes supersensitivity to the dosage of wild-type *KAR1+*, even a single extra copy of *KAR1+* is toxic in a *CDC31-16* mutant. When *DSK2-1* on a low copy plasmid was transformed into a *CDC31-16* strain, very few transformants were obtained. However, many transfromants were obtained when the *CDC31-16* mutant was transformed by either the plasmid vector or a low copy plasmid containing *DSK2+*. Furthermore, many transformants were obtained when an equivalent amount of the *DSK2-1* plasmid was transformed into the *CDC31* wild-type strain. Therefore, the *DSK2-1* allele was toxic in the presence of the *CDC31-*

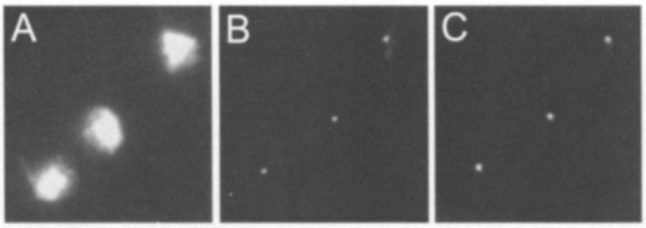


Figure 10. DSK2-1 relocalizes Cdc31p to the SPB in a *kar1* mutant strain. Indirect immunofluorescence was performed on a *kar1* deletion strain kept alive by the *DSK2-1* suppressor. The anti-Cdc31p staining (*B*) corresponds to the anti-90-kD staining (*C*) on the nucleus as determined by DAPI staining (*A*).

16 allele, in the same fashion as increased dosages of *KAR1+* (Vallen et al., 1994). We also tested the toxicity of *DSK2-1* to other *cdc31* mutant alleles and found that it is toxic to *cdc31-2* but not to *cdc31-1* or *cdc31-5*. Strikingly, the *CDC31-16* and *cdc31-2* alleles both map to the COOH-terminus of Cdc31p, a region previously identified as critical to *CDC31* suppression of *kar1Δ17* (Vallen et al., 1994). *DSK2-1* did not suppress the temperature sensitivity of any of the *cdc31* mutants, supporting the hypothesis that Cdc31p acts downstream of Dsk2p.

Dsk2p Is a Nuclear-enriched Protein

Since *DSK2-1* restores Cdc31p localization to the SPB in a *kar1* deletion, we wanted to determine whether it is a SPB component that interacts with Cdc31p. Affinity-purified anti-Dsk2p antibodies were used for indirect immunofluorescence on wild-type and *dsk2Δ* strains. DAPI staining was used to visualize the nucleus, and anti-tubulin staining was used to visualize the microtubules. Cells were processed by both formaldehyde fixation and the methanol-acetone method of Rout and Kilmartin (1990) and showed the same result. Dsk2p staining appeared to be nuclear enriched (Fig. 11 *A*, *right*), as determined by colocalization with the DAPI staining in wild-type cells (Fig. 11 *A*, *middle*) and the lack of staining in the *dsk2* deletion cells (Fig. 11 *B*, *right*) In addition, there is a cytoplasmic pool of Dsk2p. That the nuclear staining was not due to bleedthrough of the DAPI staining was demonstrated by performing anti-Dsk2p staining without DAPI staining (data not shown). To determine if the localization of Dsk2-1p might be different than the wild-type protein, indirect immunofluorescence was also performed on a *DSK2-1* strain. Like the wild-type protein, Dsk2-1p was a nuclear protein (data not shown).

To determine whether Dsk2p might be a SPB component that is not detectable by immunofluorescence due to the large nuclear pool of Dsk2p, a Western blot was performed on partially purified SPBs. Equal amounts of each fraction of the purification (see Materials and Methods for details) were Western blotted with anti-Dsk2p (Fig. 11 *D*) and anti-Cdc31p (Fig. 11 *C*) antibodies. The Dsk2 protein appeared enriched in the nuclear fraction (Fig. 11 *D*, lanes *6* and *7*), but it was not detectable in the SPB fractions (Fig. 11 *D*, lanes *12* and *13*). However, Cdc31p, a component of the SPB (Spang et al., 1993; Biggins and Rose, 1994), was detectable in both the nuclear (Fig. 11 *C*, lanes *6* and *7*) and SPB fractions (Fig. 11 *C*, lanes *12* and *13*). We

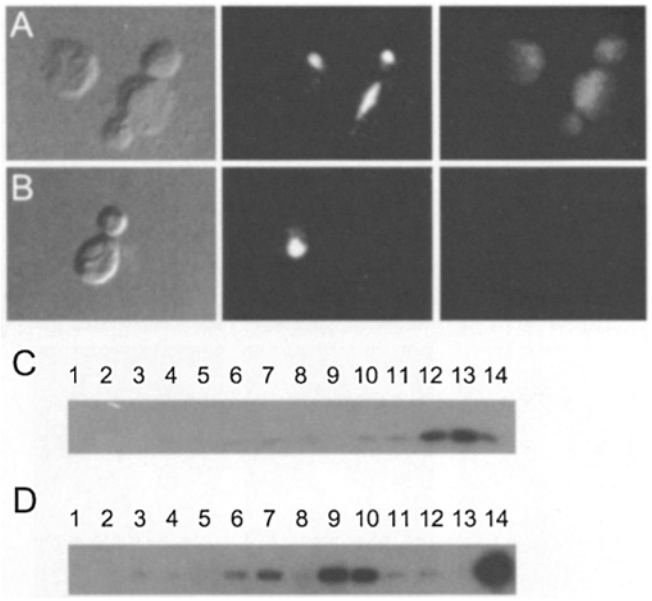


Figure 11. Dsk2 is a nuclear-enriched protein. (*A* and *B*) Indirect immunofluorescence was performed on wild-type (*A*) and *dsk2Δ* (*B*) strains using affinity-purified anti-Dsk2p antibodies. Nomarski optics are shown in the left panels, DAPI staining in the middle panels, and anti-Dsk2p staining in the righthand panels. Dsk2p stains the entire cell and is enriched in the nucleus as determined by the increased staining that corresponds to the DAPI staining. This staining is not present in the *dsk2Δ* strain, indicating it is specific to the Dsk2p protein. (*C* and *D*) Western blots on partially purified SPBs. Western blots using anti-Cdc31p (*C*) and anti-Dsk2p (*D*) were performed on partially purified SPBs. The fraction numbers of gradients from the purification are indicated above the blots. Yeast spheroplasts were prepared and lysed (lysate is shown lane *1*). A crude nuclear pellet, (pellet, lane *3*; supernatant, lane *2*) was loaded onto a gradient of layers 2.0, 2.1, and 2.3 M sucrose-polyvinylpyrolidone. The fractions from this gradient are: lane *4* (sample layer), lane *5* (sample layer/2.01 M), lane *6* (2.01/2.1 M), lane *7* (2.1/2.3 M), and lane *8* (2.3 M). The nuclei from the 2.1/2.3 fraction were collected and lysed, and SPBs were subsequently enriched on a second sucrose gradient (lanes *9–13*). The fractions from this gradient are: lane *9* (sample layer), lane *10* (sample layer/1.75 M), lane *11* (1.75/2 M), lane *12* (2/2.25 M), and lane *13* (2.25/2.5 M). The nuclei are present in fractions 6 and 7, nuclear envelope is in fractions 9 and 10, and the SPBs are in fractions 12 and 13. Cdc31p is enriched in the SPB fractions, while Dsk2p is enriched in nuclei but not in SPBs. Total yeast lysate as a control for antibody staining is shown in lane *14*.

conclude that Dsk2p is a nuclear-enriched protein that is probably not a component of the SPB.

Discussion

Dsk2 Is a Ubiquitin-like Protein

We cloned the *DSK2* suppressor of *kar1Δ17* by suppression of the temperature-sensitive defect and found it encodes a novel ubiquitin-like protein containing an NH_2-terminal domain homologous to ubiquitin and a novel COOH-terminal domain. Ubiquitin-like proteins have been identified in many other organisms, such as the human *BAT2* and *BAT3* genes in the major histocompatibility complex (Banerji et al., 1990). However, although numer-

ous ubiquitin-like proteins have been identified, their mechanism of action remains unknown.

We determined that like Rad23p, the NH_2-terminal domain of Dsk2p is not cleaved from the protein. Therefore, Dsk2p cannot be posttranslationally conjugated to substrate proteins like ubiquitin to cause substrate degradation or modification. Since the *DSK2* suppressor mutations mapped to the ubiquitin-like domain, we tested whether this domain was sufficient for *DSK2* suppression of *kar1Δ17*. Although the ubiquitin-like domains were expressed at levels comparable to the full-length protein (data not shown), neither the *DSK2+* nor *DSK2-1* ub-like domain could suppress *kar1Δ17*. Therefore, unlike ubiquitin, the COOH-terminal domain of Dsk2p is required for protein function.

The *DSK2-1* and *DSK2-2* suppressor mutations of *kar1Δ17* both changed amino acid 69 from histidine to tyrosine in the ubiquitin-like domain. This residue, which corresponds to amino acid 68 in ubiquitin, is conserved in ubiquitin and is important for the proteasomal degradation of polyubiquitinated substrates (Ecker et al., 1987). In a previous study, a mutation in this residue in ubiquitin decreased the proteolysis rate of substrates to 30% of wild type (Ecker et al., 1987). Dsk2p and Cdc31p have extremely long half-lives, and the *DSK2-1* mutation did not alter the stability of these proteins. In addition, we could not detect ubiquitinated forms of either Cdc31p or Dsk2p (data not shown), indicating that these proteins probably are not degraded by the ubiquitin pathway. Therefore, if the *DSK2-1* mutation alters proteolysis, it must change the stability of an unknown protein.

DSK2 Is Involved in SPB Duplication

Since a *dsk2* deletion has no obvious phenotype, we tested whether this is due to functional redundancy with the other ubiquitin-like gene in yeast, *RAD23*, originally isolated in a screen for UV-sensitive mutants. A *dsk2 rad23* double deletion strain is temperature sensitive due to a defect in SPB duplication. In contrast to the *kar1Δ17* allele, which is defective for the maintenance of Cdc31p localization at the SPB (Biggins and Rose, 1994), Cdc31p was still localized at the single SPB. However, since *CDC31-16* and high copy *CDC31+* can suppress the temperature sensitivity of *rad23Δ dsk2Δ*, their function must be mediated in concert with Cdc31p. In addition, since *CDC31* alleles suppress the *dsk2Δ* rad23Δ strain and *DSK2* alleles cannot suppress *cdc31* mutants, *CDC31* must function downstream of these genes for SPB duplication.

Unlike the *rad23* mutant, the *dsk2Δ* does not exhibit UV sensitivity. In addition, in a *dsk2 rad23* double deletion strain, UV sensitivity was equivalent to *rad23Δ*, so it is unlikely that they share functional redundancy for DNA repair. Therefore, we propose that the two known yeast ubiquitin-like proteins are involved in SPB duplication, and *RAD23* has an additional role in DNA repair.

In addition to the defect in SPB duplication when *dsk2* and *rad23* are deleted, we found that overexpression of *DSK2* and *DSK2-1* causes toxicity. The overexpression of *DSK2+* causes cells to accumulate with short bipolar spindles, while overexpression of *DSK2-1* causes a block in SPB duplication. Therefore, the overexpression of the mutant *DSK2-1* causes an earlier cell cycle block than overexpression of wild-type *DSK2*. Since neither loss of function nor gain of function *dsk2* mutants lead to a perfectly uniform arrest in the cell cycle, Rad23p/Dsk2p may have multiple roles throughout the cell cycle. This hypothesis is consistent with the finding that wild-type and *DSK2-1* overexpression block at different stages.

The overexpression of *KAR1+* (Rose and Fink, 1987) also causes toxicity when overexpressed, probably due to the formation of a large aggregate that contains Kar1p and Cdc31p (Biggins and Rose, 1994). Although overexpressed Dsk2p forms aggregates throughout the cell, we could not detect the presence of Cdc31p in these aggregates (data not shown). Therefore, although we did not determine the mechanism by which overexpression causes a block in SPB duplication, the dosage of *DSK2* is critical for accurate SPB duplication.

Models for DSK2 Suppression of kar1

Like the *CDC31* suppressors of *kar1Δ17*, the *DSK2-1* suppressor restores the localization of Cdc31p to the SPB in a *kar1* mutant. Dsk2-1p may relocalize Cdc31p to the SPB by either directly physically interacting with Cdc31p or by indirectly assisting Cdc31p localization. Although Dsk2p is a nuclear protein, it might transiently interact with the SPB to localize the Cdc31 protein. We used several techniques to determine whether Dsk2p and Cdc31p interact physically. First, no binding was observed in a gel overlay assay in which radiolabeled Cdc31p was used to probe immobilized bacterial and yeast Dsk2p and Dsk2-1p (data not shown; Biggins and Rose, 1994). Second, Dsk2p, Dsk2-1p, and Cdc31p did not coimmunoprecipitate from yeast. Finally, Dsk2p and Cdc31p did not coaggregate in vivo into a complex formed by overexpression of Dsk2p (data not shown). Therefore, Dsk2p and Cdc31p may transiently interact in vivo but probably do not form a stable complex.

We previously proposed that *CDC31* suppressors may bypass *KAR1* function due to increased interaction with another unknown protein (Vallen et al., 1994; Biggins and Rose, 1994). Here we showed that *DSK2* function is not required for *CDC31-16* to suppress *kar1Δ17*. However, a *kar1Δ17 dsk2Δ rad23Δ* strain is very temperature sensitive, indicating that these three genes affect a common function. Because both *CDC31-16* and high copy *CDC31+* can suppress the temperature sensitivity of this strain, the *kar1Δ17 dsk2Δ rad23Δ* strain is specifically defective for *CDC31* function. Furthermore, neither Dsk2p nor Rad23p are required for Cdc31p suppression of *kar1Δ17*. Therefore, as is the case for *KAR1*, *CDC31* gene function must be downstream of *DSK2*, and there must be at least one additional factor with which Cdc31p suppressors show an altered interaction to suppress *kar1* mutations. The *CDC31-16* allele is sensitive to the dosage of *DSK2-1*, just as it is sensitive to the dosage of *KAR1+*. In addition, *DSK2-1* is also toxic to the *cdc31-2* allele, another COOH-terminal *cdc31* mutation. This region was previously determined to be critical to the *CDC31* mechanism of *kar1Δ17* suppression (Vallen et al., 1994). Therefore, *CDC31* is sensitive to the dosage of *DSK2*, suggesting some type of interaction between the *DSK2* and

CDC31 genes. Taken together, these data suggest that *DSK2* mediates most of its functions through *CDC31*.

Dsk2p is a novel ubiquitin-like protein that has a role in SPB duplication. The *DSK2-1* suppressor exhibits many phenotypes similar to the *KAR1* gene and can relocalize Cdc31p to the SPB in a *kar1* deletion strain. Since Dsk2p is a nuclear-enriched protein that acts upstream of *CDC31* in the SPB duplication pathway, we propose that Dsk2p may have a chaperone-like role similar to Rad23p and assist Cdc31p assembly into a new SPB. Since the *dsk2Δ rad23Δ* strain that blocks SPB duplication at 37°C did not affect the localization of Cdc31p at the sole SPB, *DSK2* function may not affect the old SPB. Therefore, we propose that Dsk2p is involved in the assembly of a new SPB, and Rad23p may be either directly involved in this process or else be able to functionally substitute for Dsk2p.

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