The Calcium-binding Protein Cell Division Cycle 31 of *Saccharomyces cerevisiae* Is a Component of the Half Bridge of the Spindle Pole Body

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Abstract. *Cdc31* mutants of *Saccharomyces cerevisiae* arrest at the nonpermissive temperature with large buds, G2 DNA content and, a single, abnormally large spindle pole body (SPB) (Byers, B. 1981. Molecular Genetics in Yeast. Alfred Benzon Symposium. 16:119–133). In this report, we show that the *CDC31* gene product is essential for cell viability. We demonstrate that purified CDC31 protein binds Ca^{2+} and that this binding is highly specific. Taken together, three lines of evidence indicate that CDC31 is a component of the SPB. First, CDC31 cofractionates with enriched preparations of SPBs. Second, immunofluorescence staining indicates that CDC31 colocalizes with a known SPB component. Third, immunoelectron microscopy with whole cells and with isolated SPBs reveals that CDC31 is localized to the half bridge of the SPB, which lies immediately adjacent to the SPB plaques. CDC31 was detected mainly at the cytoplasmic side of the half bridge and, therefore, defines a further substructure of the SPB. We suggest that CDC31 is a member of a family of calcium-binding, centrosome-associated proteins from a phylogenetically diverse group of organisms.

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In the budding yeast *Saccharomyces cerevisiae*, centrosomal functions are provided by the spindle pole body (SPB),[1] a cylindrical multilaminated organelle which is embedded in the nuclear envelope (Byers and Goetsch, 1975; see Fig. 8). An outer, central, and inner plaque can be discriminated by EM. The central plaque serves to anchor the SPB in the nuclear envelope. Outer and inner plaques function as nucleation sites for cytoplasmic and nuclear microtubules, respectively (Byers, 1981*a*). The inner plaque is not as electron dense as the outer and central plaques and is only clearly detectable after extraction of isolated SPBs with DEAE-dextran (Rout and Kilmartin, 1990). An additional substructure of the SPB, the half bridge, appears in the electron microscope as a darkly stained strip along the cytoplasmic margin of the nuclear envelope (Byers and Goetsch, 1975; Byers, 1981*a*,*b*).

The SPB, in common with the centrosomes of higher eukaryotes, shows cell cycle-dependent behavior (Byers and Goetsch, 1975; Byers, 1981*a*,*b*). In G1 of the cell cycle, the single SPB in each yeast cell is duplicated. Two stages can be distinguished. First, a structure called the satellite forms on the cytoplasmic face of the half bridge. During the second phase, the satellite disappears, and two SPBs can be observed, side by side in the nuclear envelope. The duplicated SPBs are connected by a complete bridge which is later severed as the SPBs undergo separation to form the poles of a mitotic spindle. After chromosome segregation, nuclear division, and cytokinesis, each yeast cell retains exactly one SPB.

Despite the fact that the SPB plays such a vital role throughout the life cycle of the yeast cell, relatively little is known about its subunit composition and cell cycle regulation. In recent years, these problems have been addressed by both biochemical and genetic analysis. An enrichment procedure for SPBs has been reported by Rout and Kilmartin (1990). Spindle or SPB components with molecular weights of 80, 90, and 110 kD were identified by raising mAbs against enriched SPB fractions. In addition, a number of mutants defective in SPB functions have been isolated and characterized. *KAR1* was first identified as a gene involved in karyogamy (Conde and Fink, 1976). Subsequent studies have provided evidence that KAR1 is a SPB component with domains important for karyogamy and SPB duplication (Rose and Fink, 1987; Vallen et al., 1992*a*,*b*). The product of the *CIK1* gene has also been implicated as a possible component of the SPB; CIK1 is found to localize with SPBs after treatment of yeast cells with mating pheromone (Page and Snyder, 1992). A mutant of *MPS1* arrests at the nonpermissive temperature as large-budded cells with a monopolar spindle (Winey et al., 1991). Such cells were found to possess an enlarged half bridge adjacent to the single SPB. Strains mutant in *MPS2* undergo an aberrant cycle of SPB duplication which gives rise to one functional SPB and one defective SPB that lacks any microtubules on its nuclear face and resides entirely in the cytoplasmic compartment (Winey et al., 1991). A cold sensitive mutant, *ndc1*, revealed a similar phenotype to *mps2* (Thomas and Botstein, 1986). Re-

1. *Abbreviations used in this paper*: CDC, cell division cycle; SPB, spindle pole body.

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cently, it has been shown that mutations in yeast calmodulin (CaM) also cause defects in SPB function and nuclear integrity. The mutations investigated by Sun et al. (1992) were found to block SPB duplication. However, the morphology of the unduplicated SPB appeared unaffected, suggesting that CaM functions at an early stage of SPB duplication.

Of particular interest is the cell division cycle (*CDC*) gene *CDC31*. When cells of most *cdc* mutants are transferred to the nonpermissive temperature, the SPB cycle arrests at a stage which reflects other markers of cell cycle progression, e.g., DNA replication and bud formation. Mutations in *CDC31*, however, cause an uncoupling of SPB duplication from cell cycle control. In *cdc31* strains subjected to the nonpermissive temperature, DNA replication proceeds despite a failure in SPB duplication, and the cells undergo a mitosis in which all the chromosomes segregate to the single functional SPB, resulting in diploidization of the single surviving daughter cell (Byers, 1981*a*; Schild et al., 1981). Electron microscopic analysis of such cells reveals a single SPB which is abnormally large and lacks a satellite (Byers, 1981*b*). The *CDC31* gene has been cloned and sequenced and found to encode a small, acidic protein of 161 amino acids with a striking homology to Ca^{2+}-binding proteins, e.g., calmodulin or caltractin (Baum et al., 1986; Huang et al., 1988). Centrin, which is closely related to caltractin, has been identified as a component of the basal body of the green alga *Tetraselmis striata* (Salisbury et al., 1984). Interestingly, a number of centrosomal proteins, heterogenous in molecular weight and with immunogenic epitopes related to centrin, have been identified in several different organisms (Moudjou et al., 1991; Baron and Salisbury, 1988; Huang et al., 1988). These results define a heterogenous group of calcium-binding proteins that associate with centrosomes from phylogenetically diverse organisms.

In this publication we show that CDC31 is a further member of this protein family. First, CDC31 binds Ca^{2+}, and this binding is highly specific. Second, CDC31 is a component of the yeast SPB. The protein is associated with the cytoplasmic side of the SPB half bridge. In addition, we show that CDC31 is essential for the viability of yeast cells which emphasizes the importance of calcium binding proteins for centrosomal functions.

Materials and Methods

Yeast Strains, Media, DNA Techniques, Bacterial and Yeast Transformation

Table I summarizes the yeast strains that were used in this study. The media for growth of yeast were prepared as described by Guthrie and Fink (1991). The standard yeast culture medium used was yeast extract, peptone, dextrose growth medium (YPD). All DNA manipulations were performed as described by Sambrook et al. (1989). Yeast (Grey and Brendel, 1992) and *E. coli* (Dower et al., 1988) strains were transformed by electroporation.

Cloning of CDC31

CDC31 was cloned by PCR using the following primers, which were complementary to the published sequence of Baum et al. (1986): Primer CDC31-I: 5′-TAACCCAAAGCTTGATGTATTTCTCTAGTA-3′, CDC31-II: 5′-TGTTGCCCTGCAGGATTAGATTGGCTTTTT-3′, CDC31-IV: 5′-GAACTGCGGCCGCTACTATCGGTGCAAATAGCTATAAA-3′. Combinations of primers CDC31-I/II and CDC31-I/IV were used. The PCR was performed with Taq polymerase supplied by Perkin-Elmer Corp. (Norwalk, CT). Chromosomal DNA prepared from strain S288C was used as a template. The PCR product was cloned into the HindIII–PstI or HindIII–NotI sites of vector pBluescript SK (Stratagene, La Jolla, CA). The plasmids were named pSM1 and pBM6, respectively. The nucleotide sequence of the cloned PCR products was determined by the chain-termination method of Sanger et al. (1977). The HindIII–PstI fragment of pSM1 carrying the *CDC31* gene was cloned into the centromere-based yeast–*E. coli* shuttle vectors pRS315 and pRS316 (Sikorski and Hieter, 1989). The resulting plasmids were named pSM17 and pUF7.

Construction of a CDC31 Gene Disruption

CDC31 was disrupted by the one-step gene replacement method of Rothstein (1983). A fragment 5′ of the coding region of *CDC31* was amplified by PCR with primers CDC31-I and CDC31-Pst: 5′-TGGACCAGACTG-CAGCGATGACCT-3′. HindIII and PstI restriction endonuclease cleavage sites were incorporated into the sense and antisense PCR primers, respectively, facilitating subcloning of the PCR product into the multiple cloning site of plasmid pBluescript SK. The recombinant plasmid generated was named pSM6. A PstI/SacI fragment 3′ of the *CDC31* coding region was ligated into the PstI and SacI restriction sites of plasmid pSM6, generating plasmid pSM7. The *HIS3* gene on a 1,157-bp PstI fragment was inserted into the PstI site of pSM7. The resulting plasmid pUF6 was restricted with SacI and SalI, and the fragments were transformed into the diploid yeast strain YPH501, selection being made on Synthetic Complete (SC) Media plates lacking histidine. Chromosomal DNA from four His^+ transformants and the parental strain was isolated and digested with restriction endonuclease HindIII. The fragments of DNA were separated on an agarose gel and transferred to a Hybond-N membrane (Amersham Buchler, Braunschweig, Germany) by vacuum blotting. The HindIII–SacI fragment from pSM7 (which is deleted for the entire coding region of *CDC31*) was labeled with digoxygenin and used as a probe in Southern analysis. Preparation of labeled probe, hybridization, and detection were performed with a kit from Boehringer Mannheim (Mannheim, Germany). HindIII digested λ-DNA (Boehringer Mannheim) labeled with digoxygenin was used as a molecular weight marker.

Isolation of the CDC31 Protein

The *CDC31* gene was amplified from plasmid pSM1 by PCR using primers CDC31-ATG (5′-ATAGGCATATGAGTAAGAACAGGTCATCGC-3′) and CDC31-II. The sense primer was designed such that the translation initiation codon (ATG) of *CDC31* formed part of an NdeI restriction site. The product of the PCR was subcloned into the NdeI and PstI restriction sites of expression vector pT7-7 (Tabor and Richardson, 1985), generating a recombinant plasmid, named pSM5, in which expression of CDC31 was under the control of the inducible phage T7 promoter. Expression of the CDC31 protein was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to a mid-log phase culture of BL21 (DE3, pSM5) (Studier and Moffat, 1986). The cells were harvested after 3 h of induction and were lysed by ultrasonication. Unlysed cells and inner and outer *E. coli* membranes were removed by ultracentrifugation (1 h, 100,000 *g*, 4°C). The supernatant fraction was loaded on to a DE52 (Whatman, Kent, England) anion exchange column. The column was equilibrated with buffer C (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT). Proteins were eluted by a linear salt gradient from 50 to 500 mM NaCl. Fractions containing CDC31 (0.1 and 0.15 M NaCl) were pooled and loaded on to a hydroxyapatite column (Bio-Gel HT; Bio-Rad, Richmond, CA), equilibrated with buffer C. After loading, the column was washed with three column volumes of 1 M NaCl and subsequently equilibrated with 10 mM potassium phosphate, pH 6.8. CDC31 was eluted by a linear gradient from 10 to 300 mM potassium phosphate, pH 6.8. The fractions containing CDC31 (0.15–0.2 M phosphate) were pooled and dialyzed against buffer C.

Cell Fractionation, Nuclei Isolation, SPB Preparation

Cells of the diploid yeast strain BJ5626 were spheroplasted and lysed according to the procedure of Rout and Kilmartin (1990). Unlysed cells were removed by low speed centrifugation (10 min, 800 *g*, 4°C). A portion of the cell extract was separated into a soluble and an insoluble fraction by ultracentrifugation (1 h, 100,000 *g*, 4°C). The bulk of the cell extract was used for the isolation of nuclei and enrichment of SPBs as described by Rout and Kilmartin (1990).

Preparation of Affinity-purified Anti-CDC31 Antibodies

The *CDC31* gene was amplified from plasmid pSM1 by PCR using primers

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Table I. Yeast Strains

Strain	Genotype	Source of reference
S288C	*MATα mal gal2*	R. Mortimer (University of California, Berkeley, CA)
BJ5626	*MATa/α ura3-52/ura3-52 trp1/+ +/leu2Δ1 his3Δ200/his3Δ200 pep4::HIS3/pep4::HIS3 prb1Δ1.6R/prb1Δ1.6R can1/can1 GAL/GAL*	B. Jones (Carnegie Mellon University, Pittsburgh, PA)
YPH501	*MATα/a ura3-52/ura3-52 lys2-801^{amber}/lys2-801^{amber} ade2-101^{ochre}/ade2-101^{ochre} trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1*	Sikorski and Hieter (1989)
FY78	*MATa his3Δ200*	F. Winston (Harvard Medical School, Cambridge, MA)
UFM1	*MATα/a ura3-52/ura3-52 lys2-801^{amber}/lys2-801^{amber} ade2-101^{ochre}/ade2-101^{ochre} trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 CDC31/Δcdc31::HIS3*	this study
BMY11	*MATα ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1Δ63 his3Δ200 leu2Δ1 Δcdc31::HIS3* {pSM17*}	this study
BMY12	*MATα ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1Δ63 his3Δ200 leu2Δ1 Δcdc31::HIS3* {pSM17*, pUF7‡}	this study
BMY13	*MATα ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1Δ63 his3Δ200 leu2Δ1 Δcdc31::HIS3* {pSM17-1*, pRS315§}	this study
BMY14	strain YPH501 harboring plasmid pRS316‖	this study

The strain harbors the autonomously replicating, centromere-based plasmid whose name is indicated in brackets.
* pSM17-1: *CDC31* was inserted into pRS316.
‡ pUF7: *CDC31* was inserted into pRS315.
§ pRS315 is a *LEU2*-based plasmid (Sikorski and Hieter, 1989).
‖ pRS316 is a *URA3*-based plasmid (Sikorski and Hieter, 1989).

CDC31-Bam: 5′-GCTGGGGATCCGTAAGAACAGGTCATCGCT-3′ and CDC31-EcoRI: 5′-TCGAAGAATTCAAGTGCATCCTTGGGCAAG-3′. Subcloning the PCR product, restricted with BamHI and EcoRI, into the multiple cloning site of plasmid pGEX-3X (Pharmacia Biotech Inc., Piscataway, NJ) resulted in an in-frame fusion of the *CDC31* coding region downstream of glutathione-S-transferase (GST). The recombinant plasmid was named pSM4. Expression and purification (using a Glutathione Sepharose 4B column) of the fusion protein was as recommended by Pharmacia. Antibodies against the GST-CDC31 fusion protein were raised in rabbits as described by Harlow and Lane (1988). Affinity-purified anti-CDC31 antibodies were prepared by affinity chromatography as described by Harlow and Lane (1988). The IgG fraction containing anti-CDC31 antibodies was incubated with purified CDC31 coupled to CNBr-Sepharose 4B (Pharmacia LKB, Freiburg, Germany). Anti-CDC31 antibodies were eluted with 100 mM glycine, pH 2.5. The pH was immediately adjusted to pH 7.5 with 1 M Tris-HCl, pH 8.0. The antibodies were adsorbed on 1/3 volume of heated (1 h, 70°C) BJ5626 yeast cells for 1 h at 4°C. The affinity-purified anti-CDC31 antibodies gave a positive signal with CDC31, but not with GST, on an immunoblot.

Immunoelectron Microscopy, Immunofluorescence of Nuclei

Immunoelectron microscopy of whole yeast cells was performed as described by Preuss et al. (1991). Immunoelectron microscopy of SPBs were performed as described by Pipe (1986). A 1:10 dilution of affinity-purified anti-CDC31 antibodies was used. The gold particles were visualized in a Jeol JEM 100B electron microscope (Jeol Ltd., Tokyo, Japan).

Indirect immunofluorescence of isolated nuclei was performed by a modification of the method of Adams and Pringle (1984). Isolated yeast nuclei from strain BJ5626 were resuspended in buffer A (40 mM potassium phosphate, pH 6.5, 0.5 mM $MgCl_2$, 1.2 M sorbitol) and incubated with 2% formaldehyde for 30 or 60 min. Nuclei were sedimented by centrifugation (10 min, 150,000 *g*, 4°C), resuspended in buffer A, and overlaid onto glass cover slips pretreated with polylysine. The nuclei were allowed to settle at room temperature for 1 h and were then incubated with primary antibody for 2 h. After washing and incubation with the secondary antibody for 2 h, the DNA was stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI). No signal was obtained when nuclei were incubated either with secondary antibody only or with preimmune serum. Similarly, no signal was obtained in double-labeling experiments in which treatment with either of the primary antibodies was followed by incubation with the "mismatched" secondary antibody. Immunofluorescence microscopy was performed with a Zeiss Axiophote microscope (Carl Zeiss, Inc., Thornwood, NY). A pool of various mouse monoclonal anti-90-kD antibodies was a generous gift from Dr. J. Kilmartin. Monoclonal rat antibody against yeast tubulin (YOL1/34) was from Sera-lab. Secondary antibodies were goat anti-rabbit IgG conjugated with Cy3, goat anti-mouse IgG conjugated with FITC, and goat anti-rat IgG conjugated with FITC from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunoblotting, Protein Determination

The protein content of samples was determined by the method of Bradford (1976). Proteins were separated by SDS-PAGE according to the method of Laemmli (1970) or Brundage et al. (1990). Immunoblotting was performed as described by Harlow and Lane (1988). Primary antibodies were as described in the legends. In the case of the mouse anti-90-kD and anti-110-kD mAbs, incubation with primary antibody was followed by an incubation with rabbit anti-mouse IgG. Secondary antibodies were goat anti-rabbit IgG conjugated with horseradish peroxidase from Bio-Rad (Bio Rad Laboratories, Hercules, CA). Detection was made by addition of 3,3′-diaminobenzidine (DAB) in the presence of $NiCl_2$ (Tsang et al., 1985) or by enhanced chemiluminescence (ECL) using a kit from Amersham Buchler GmbH (Braunschweig, Germany).

Results

The CDC31 Gene Product Is Essential for Cell Viability

CDC31 was cloned from yeast strain S288C by PCR using primers complementary to the published sequence (Baum et al., 1986). Analysis of the nucleotide sequence of the cloned *CDC31* gene revealed three discrepancies with the published sequence. Each of these discrepancies was confirmed by three independent cloning and sequencing experiments. An insertion of four nucleotides (GGAA) was found following

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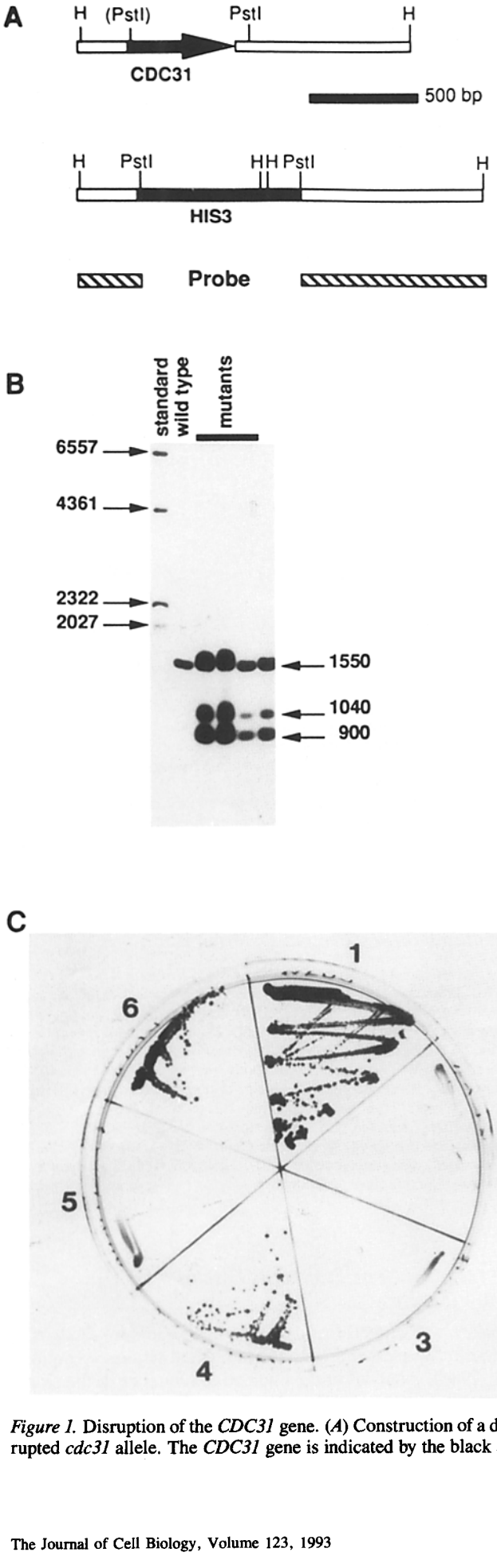

Figure 1. Disruption of the *CDC31* gene. (*A*) Construction of a disrupted *cdc31* allele. The *CDC31* gene is indicated by the black arrow. An additional PstI site, shown in parentheses, was introduced into the 5′ noncoding region of *CDC31* by PCR. The PstI fragment containing 95% of the *CDC31* coding region was substituted by a fragment of DNA carrying the *HIS3* gene. Details of the construction are described in Materials and Methods. (*B*) Southern hybridization. Genomic DNA prepared from the parental strain YPH501 and from four putative mutants was digested with HindIII, and the restriction fragments were separated by agarose gel electrophoresis. The DNA probe used for hybridization analysis consisted exclusively of sequences 5′ and 3′ of the *CDC31* gene. DNA fragment sizes are shown in base pairs. HindIII restriction sites are abbreviated by the letter H. (*C*) *CDC31* is an essential gene. Yeast strains were incubated at 30°C on a SC plate containing 5-FOA. (*1*) UFM1 [*CDC31, Δcdc31::HIS3, ura3, ura3*]; (*2*) FY78 (*URA3*); (*3*) BMY11 (*Δcdc31::HIS3, ura3*; a segregant of UFM1 carrying plasmid pSM17 [*CDC31, URA3*]); (*4*) BMY12 [BMY11 carrying plasmid pUF7 (*CDC31, LEU2*)]; (*5*) BMY13 (BMY11 carrying plasmid pRS315 [*LEU2*]); and (*6*) YPH501 carrying plasmid pRS316 (*URA3*). The growth behavior of control strains (*1*, *2*, and *6*) indicated that selection on the 5-FOA-containing plates was working.

nucleotide −37 (numbering as in Baum et al., 1986) in the 5′ noncoding region of *CDC31*. Two other discrepancies (a TT to AA transversion at positions 229 and 230 and a TC to CT transition at positions 329 and 330) were located in the coding region of *CDC31* and resulted in the amino acid substitutions Leu77Lys and Ile110Thr. It was clear from the results of complementation experiments (see for example Fig. 1 *C*) that our cloned *CDC31* gene encoded a functional protein. We assume, therefore, a polymorphism in the *CDC31* nucleotide sequence from different yeast strains.

To investigate whether *CDC31* is essential for cell viability, a gene disruption experiment was performed. A derivative of the diploid yeast strain YPH501 was isolated in which one of the two wild-type alleles of *CDC31* was disrupted with a fragment of DNA carrying the *HIS3* gene. The construction of this strain is summarized in Fig. 1 *A*. Chromosomal DNA from the parental strain and from four putative mutants was isolated and analyzed by Southern hybridization (Fig. 1 *B*). All four putative mutants contained DNA-fragments of 900 and 1,040 bp in addition to the 1,550-bp fragment characteristic of the parental strain, indicating that in each case one *CDC31* allele had been disrupted while the second allele remained wild type. One of the mutant strains, named UFM1, and parental strain YPH501 were sporulated, and the viability of spores from 20 tetrads was determined. In the case of YPH501, all four spores of each tetrad were viable. In all 20 tetrads of UFM1, however, only two of the four spores from each tetrad formed viable colonies. Furthermore, all of the viable colonies derived from UFM1 were His^-, indicating that they carried the wild-type *CDC31* allele. Spores which had failed to form viable colonies were examined under the microscope. In each case, microcolonies consisting of 2–4 enlarged cells with large buds were observed at sites of no visible colony growth (data not shown). Presumably, spores with the disrupted *cdc31* allele germinated and contained sufficient CDC31 to allow a few cell divisions, but subsequent cell division was blocked by depletion of CDC31 below a critical threshold.

The following experiments confirmed that *CDC31* is essential for cell viability. Strain UFM1, with one wild-type and one disrupted *CDC31* allele, was transformed with plas-

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mid pSM17 carrying *URA3* and *CDC31*. Ura$^+$ transformants were sporulated and the auxotrophic markers of the haploid germinants were investigated. Whilst His$^-$ Ura$^+$ and His$^-$ Ura$^-$ colonies were obtained, 100% of the His$^+$ colonies were Ura$^+$. This result clearly indicated that spores containing the *Δcdc31::HIS3* disruption (His$^+$) were only viable if plasmid pSM17 (Ura$^+$), carrying a wild-type *CDC31* allele, was retained. One of the His$^+$ Ura$^+$ haploid colonies was named BMY11. 5-fluoroorotic acid (5-FOA) can be used to select for *ura3* mutants. A yeast strain with a *ura3* mutation on the chromosome and a *URA3* allele carried on a plasmid can grow on 5-FOA only after spontaneous loss of the plasmid. We used this selection to test whether the *URA3*-based plasmid pSM17, carrying the only functional *CDC31* allele of strain BMY11, could be lost. BMY11 did not form colonies on YPD plates containing 5-FOA (Fig. 1 *C*, sector *3*), indicating that *CDC31* was essential for cell viability. However, 5-FOA–resistant colonies resulting from spontaneous loss of plasmid pSM17 were obtained when a second (*LEU2*-based) plasmid, pUF7, also carrying a *CDC31* allele, was transformed into strain BMY11 (BMY12, Fig. 1 *C*, sector *4*). In a control experiment, strain BMY11 carrying the parental *LEU2*-based plasmid pRS315 (BMY13) could not form colonies on medium containing 5-FOA (Fig. 1 *C*, sector *5*) indicating that it was the *CDC31* gene, and not the *LEU2* gene, of pUF7 that was essential for cell growth. Taking together the results of the spore viability and plasmid-shuffle experiments, we conclude that the *CDC31* gene is essential for viability of *S. cerevisiae*.

CDC31 Is a Ca^{2+}-binding Protein

CDC31 shows striking primary amino acid sequence homology to the known Ca^{2+}-binding protein calmodulin (Baum et al., 1986). The following experiments were designed to investigate the Ca^{2+}-binding properties of CDC31.

The *CDC31* gene was placed under the control of a phage T7 promoter and expressed in *E. coli*. CDC31, which was present in soluble form in an *E. coli* cell extract, was purified as described in Materials and Methods. Calmodulin shows a Ca^{2+}-dependent migration behavior in SDS-PAGE, the Ca^{2+}-free form migrating at a slower rate than the Ca^{2+}-bound form (Geiser et al., 1991). We found that CDC31 behaved in a similar manner. Purified CDC31 protein was incubated with the calcium chelator EGTA to remove all bound Ca^{2+}. When analyzed by SDS-PAGE, this Ca^{2+}-free form of CDC31 migrated at a slower rate than the untreated protein (Fig. 2 *A*, compare lanes *1* and *2*). Because the mobility of other *E. coli* proteins was unaffected by an equivalent concentration of EGTA (data not shown), we conclude that this effect is specific to CDC31. The addition of Ca^{2+} to a sample of purified CDC31 did not affect the mobility of the protein in SDS-PAGE, indicating that CDC31 had been purified in its Ca^{2+}-bound form (Fig. 2 *A*, lane *3*). Identical results were obtained with a total yeast extract in which CDC31 was detected by immunoblotting (data not shown).

Binding studies with the isotope $^{45}[Ca^{2+}]$ confirmed that CDC31 is a Ca^{2+}-binding protein (Fig. 2 *B*). Molecular weight markers (Fig. 2 *B*, lane *3*), purified CDC31 (lane *4*), and an *E. coli* extract containing CDC31 (lane *5*) were immobilized on to a nylon membrane, incubated with $^{45}[Ca^{2+}]$, and an autoradiograph of the membrane was made. CDC31 (lanes *4* and *5*) was labeled with $^{45}[Ca^{2+}]$. No protein in the

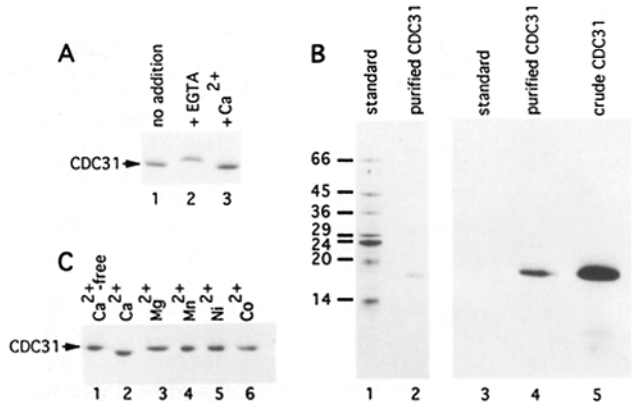

Figure 2. Binding of Ca^{2+} to CDC31. (*A*) Purified CDC31 (5 μg) in buffer B (50 mM Tris-HCl, pH 7.5, 50 mM NaCl) was incubated for 10 min at 4°C without further addition (lane *1*), or with 5 mM EGTA (lane *2*), or with 5 mM $CaCl_2$ (lane *3*). An equal volume of sample buffer was added, and the samples were analyzed by SDS-PAGE. Proteins were stained with Coomassie blue. (*B*) Molecular weight markers (lanes *1* and *3*), purified CDC31 (1 μg, lanes *2* and *4*), and an *E. coli* extract containing CDC31 (30 μg, lane *5*) were analyzed by SDS-PAGE and either stained with Coomassie blue (lanes *1* and *2*) or transferred to a nylon membrane (lanes *3*–*5*) and incubated with $^{45}[Ca^{2+}]$ as described by Geiser et al. (1991). Albumin, bovine, and egg; glyceraldehyde-3-P-dehydrogenase; carbonic anhydrase; trypsinogen; trypsin inhibitor; and lactalbumin were used as standards. Molecular weights are indicated in kD. (*C*) Purified CDC31 in buffer B was incubated with 5 mM EGTA for 10 min at 4°C. EGTA was removed by gel filtration through a G25 column equilibrated with buffer B. The discharged CDC31 was incubated without further addition (lane *1*), or with 5 mM $CaCl_2$ (lane *2*), $MgCl_2$ (lane *3*), $MnCl_2$ (lane *4*), $NiCl_2$ (lane *5*), or $CoCl_2$ (lane *6*). Samples were analyzed as in *A*.

molecular weight standards gave a positive signal (Fig. 2 *B*, compare lanes *1* and *3*). The major protein labeled in the crude *E. coli* extract (Fig. 2 *B*, lane *5*), was CDC31. The minor band in Fig. 2 *B*, lane *5* represents $^{45}[Ca^{2+}]$-binding either to a CDC31 degradation product or to intrinsic *E. coli* Ca^{2+}-binding proteins. Additional *E. coli* Ca^{2+}-binding proteins may have been undetected due to their lower abundance.

To test whether metal ion binding to CDC31 is specific for Ca^{2+}, the gel-shift assay was performed with other divalent metal ions. Bound Ca^{2+} was removed by incubation of CDC31 with EGTA. EGTA and EGTA/Ca^{2+}-complex were subsequently removed by gel filtration. Discharged CDC31 (Fig. 2 *C*, lane *1*) was incubated with various divalent metal ions and tested for ion binding by the mobility-shift assay (lanes *2*–*6*). The binding of Ca^{2+} to CDC31 was indicated by the faster migrating form of CDC31 (Fig. 2 *C*, lane *2*). However, none of the other metal ions affected the mobility of CDC31 (Fig. 2 *C*, compare lane *2* with lanes *3*–*6*).

CDC31 Cofractionates with SPBs

Mutations in *CDC31* give rise to a cell cycle defect. When incubated at the nonpermissive temperature, *cdc31* mutant cells arrest with large buds, and electron microscopic analysis reveals a single, unusually large SPB that lacks a satellite (Byers, 1981*a*). A possible explanation for the terminal phenotype of *cdc31* mutants would be that the CDC31 protein is a structural component of the SPB and directly in-

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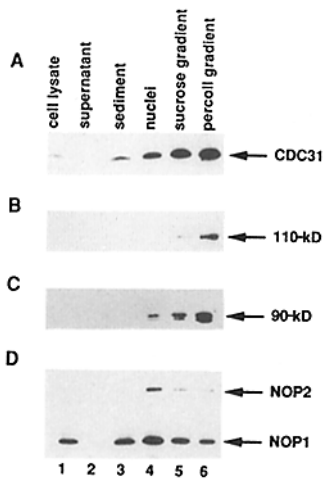

Figure 3. Cellular distribution of CDC31. A cell extract (lane *1*) from yeast strain BJ5626 was fractionated into 100,000 *g* supernatant (lane *2*) and sediment (lane *3*) fractions, isolated nuclei (lanes *4*), and enriched SPBs (lanes *5* and *6*). Fractions from the sucrose (lane *5*) and Percoll (lane *6*) gradients are progressively enriched in SPBs. Numerous SPBs were observed when thin sections of a pellet of Percoll gradient-enriched SPBs were analyzed by EM (data not shown). Equal amounts (*A*–C, 10 μg; *D*, 5 μg) of total protein per lane were analyzed by SDS-PAGE and subsequent immunoblotting using primary antibodies directed against: (*A*) CDC31, (*B*) 110-kD SPB component, (*C*) 90-kD SPB component, and (*D*) the nucleolar proteins NOP1 and NOP2. The immunoblots were developed with an ECL kit from Amersham (see Materials and Methods).

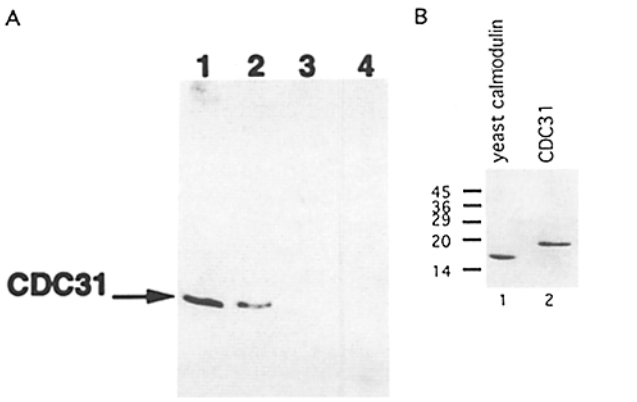

Figure 4. Affinity-purified anti-CDC31 antibodies are specific. (*A*) Total yeast extract from strain BJ5626 (lanes *1–3*) and 2 μg bovine calmodulin (lane *4*) were separated by SDS-PAGE and analyzed by immunoblotting. The following dilutions of primary antibodies were used: serum (diluted 1:300; lane *1*), affinity-purified anti-CDC31 antibodies (1:50; lanes *2* and *4*), and preimmune serum (1:10; lane *3*). The immunoblot was developed with DAB. (*B*) Purified yeast calmodulin (3 μg, lane *1*) and CDC31 (2 μg, lane *2*) were separated by SDS-PAGE and stained with Coomassie blue. The molecular weights are indicated in kD.

volved in the SPB duplication pathway. To investigate this possibility, we tested whether CDC31 cofractionates with enriched preparations of SPBs. In Fig. 3, equal amounts of total protein from a total cell lysate (lane *1*), 100,000 *g* supernatant (lane *2*) and sediment (lane *3*) fractions, isolated nuclei (lane *4*), sucrose (lane *5*), and Percoll gradient-enriched SPBs (lanes *6*) were separated by SDS-PAGE and tested for the presence of CDC31 by immunoblotting. As controls of the experimental design, the distributions of the 110-kD SPB component (Fig. 3 *B*), the 90-kD SPB component (Fig. 3 *C*), and the two nucleolar proteins NOP1 and NOP2 (Fig. 3 *D*) were also determined. As expected, the 110- and 90-kD SPB components were detected most strongly in the fractions containing purified SPBs (lanes *5* and *6*). The amounts of these proteins in Fig. 3, lanes *1–3* were in fact too little to be detected by this method. Nucleolar proteins NOP1 and NOP2 were enriched from the sediment (lane *3*) to isolated nuclei (lane *4*). As expected, the concentration of these proteins subsequently decreased with increasing purity of SPBs (compare lane *4* with lanes *5* and *6*). The results obtained with anti-CDC31 antibodies are shown in Fig. 3 *A*. Whilst a minor fraction of CDC31 (~1%) could be detected in the supernatant fraction (Fig. 3, lane *2*), the vast majority of the CDC31 (99%) was present in the sediment (lane *3*). It is clear from Fig. 3 *A* that CDC31 (like the 110- and 90-kD SPB proteins) is progressively enriched from the 100,000 *g* sediment through to the Percoll gradient-enriched SPBs (Fig. 3, lanes *3–6*). We conclude from this result that CDC31 cofractionates with purified preparations of SPBs.

CDC31 Is a Component of Spindle Pole Bodies

Results of the immunoblotting experiments indicated that the CDC31 protein is associated with SPBs. To confirm and extend our results, we used affinity-purified anti-CDC31 antibodies to investigate the cellular localization of CDC31 by indirect immunofluorescence and immunoelectron microscopy. The success of these techniques is critically dependent on the specificity of the antibody-antigen interaction. Our affinity-purified anti-CDC31 antibodies were prepared as described in Materials and Methods and their specificity was tested as shown in Fig. 4 *A*. In a total yeast extract, only one protein was recognized by the anti-CDC31 serum (Fig. 4 *A*, lane *1*) and by the affinity-purified anti-CDC31 antibodies (lane *2*), and this protein had the same migration behavior as our purified CDC31 in SDS-PAGE (data not shown). No yeast proteins were recognized by the preimmune serum (Fig. 4 *A*, lane *3*). The affinity-purified anti-CDC31 antibodies did not recognize bovine calmodulin (Fig. 4 *A*, lane *4*) or yeast calmodulin (data not shown). Bovine and yeast calmodulin are 42 and 38% identical to CDC31, respectively. In addition, yeast calmodulin (Fig. 4 *B*, lane *1*) is well resolved from CDC31 (lane *2*) by the SDS-PAGE used. These results demonstrate that our affinity-purified anti-CDC31 antibodies are highly specific for CDC31 and suitable for localization studies by indirect immunofluorescence and immunoelectron microscopy.

Staining of the 90-kD component was used as a marker of the SPB in the immunofluorescence experiments; it has previously been reported that the epitopes recognized by the anti–90-kD antibodies (a pool of mAbs) were more stable to fixation with formaldehyde than the epitopes recognized by the anti–110-kD antibodies (Rout and Kilmartin, 1990). Double-labeling experiments were performed with affinity-purified anti-CDC31 and anti–90-kD antibodies. DNA was stained with DAPI. Isolated nuclei rather than whole yeast cells were used for indirect immunofluorescence. Compared with whole cells, nuclei gave a much stronger staining of the two components, probably due to the increased accessibility of the antigen. We found that both CDC31 and the 90-kD protein were detected as one or two dots at the nuclear periphery (Fig. 5 *A*, compare 90 kD and CDC31 with DAPI staining). In >90% of cases, the CDC31 and 90-kD signals coincided. In a few cases, the 90-kD SPB component stained as two closely spaced dots that appeared as a single signal in the CDC31 staining suggesting, a close, but not identical, localization of the two proteins. The number of CDC31 sig-

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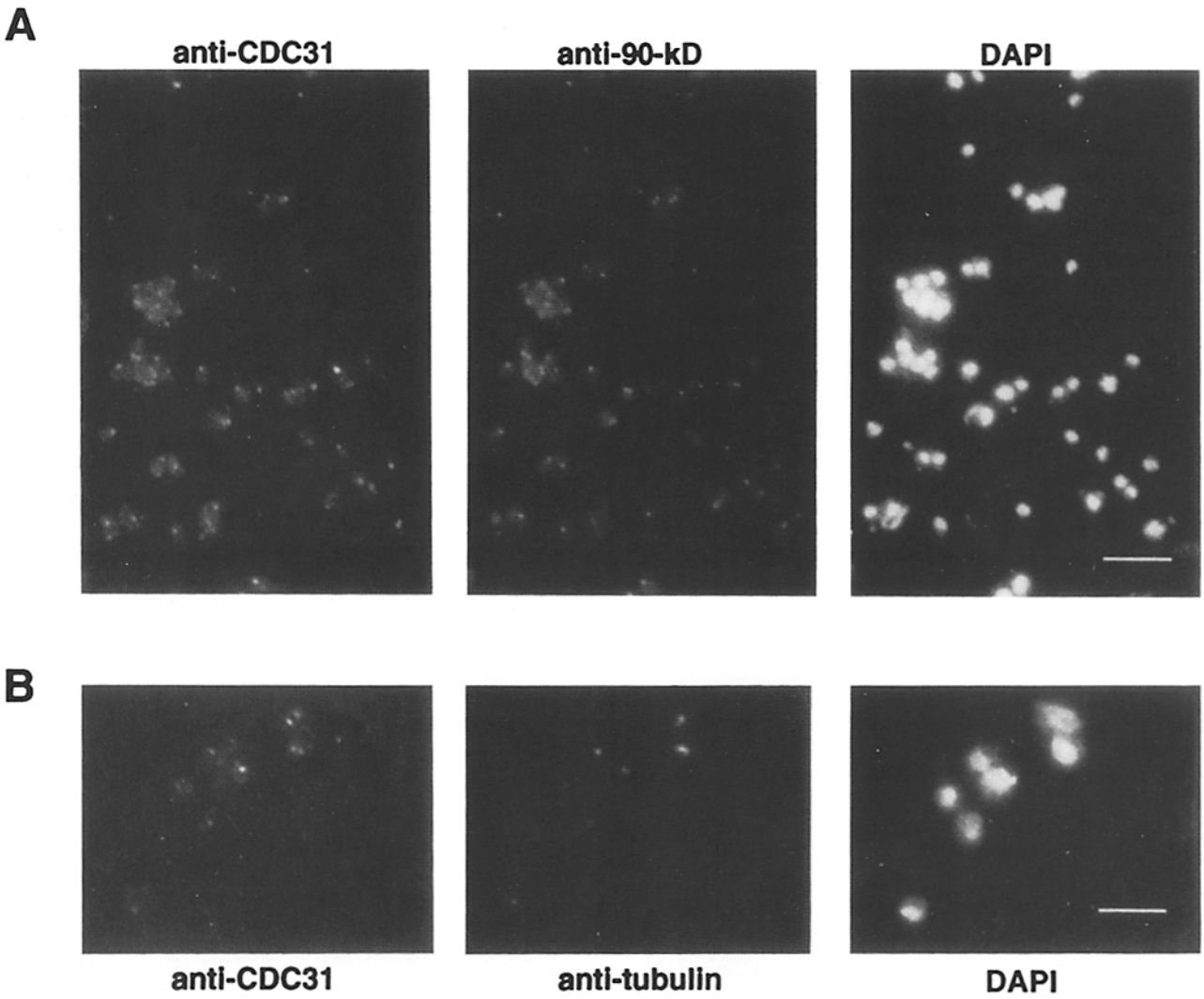

Figure 5. Immunofluorescence microscopy of isolated yeast nuclei (see Materials and Methods). (*A*) Nuclei were fixed for 30 min and then stained with affinity-purified rabbit anti-CDC31 and monoclonal mouse anti-90-kD antibodies. (*B*) Yeast nuclei, fixed for 60 min, were incubated with affinity-purified anti-CDC31 and monoclonal rat anti-tubulin antibodies. Secondary antibodies used in *A* and *B* were described in Materials and Methods. DNA was stained with DAPI. Bars: (*A*) 1.0 μm; (*B*) 1.5 μm.

nals per nucleus and their location at the nuclear periphery are entirely consistent with our observation that CDC31 is associated with the SPB complex and effectively exclude an association of CDC31 with other macromolecular structures, e.g., the nuclear pore complex or nucleolus.

As the SPB functions as a microtubule-organizing center, we tested whether CDC31 colocalizes with microtubules. Double-labeling immunofluorescence experiments were performed with isolated nuclei using affinity-purified anti-CDC31 and anti-tubulin antibodies (Fig. 5 *B*). Tubulin stained as dots or short spindles, while CDC31 staining was as described above. In some nuclei, no tubulin signal was obtained, suggesting that some microtubules depolymerized during the isolation of nuclei. Consistent with a localization of CDC31 with the SPB, the CDC31 and tubulin signals were immediately adjacent to one another, but did not coincide. In many cases where CDC31 stained as two dots, tubulin staining was observed in the region between the dots. Taken together, the results of our immunofluorescence experiments strongly suggest that CDC31 is a structural component of the SPB.

The localization of CDC31 was investigated further by immunoelectron microscopy. Ultra-thin sections of whole yeast cells were treated with affinity-purified anti-CDC31 antibodies and the bound immunoglobulins were detected by protein A labeled with colloidal gold. Only proteins which were exposed on the surface of the sections were accessible for detection by the antibodies. Since the SPB is a rather small organelle and present in only one or two copies per cell we predicted that only a minor fraction of yeast cells would show staining if CDC31 is indeed a component of the SPB. In the majority of the cells screened ($n > 1{,}000$), 2–6 gold particles were detected per cell (Fig. 6 *A*). These particles were distributed singly throughout the cell and were mostly associated with membranous organelles, e.g., vacuoles (Fig. 6 *A*, *large arrowheads*); in no cases were two gold particles located at the same site. In ~2% of the cells, clusters of gold particles were located alongside the cytoplasmic face of the nuclear envelope (Fig. 6 *A*, *small arrowhead*, and *B–F*). 3–6 gold particles were concentrated at the nuclear periphery, often associated with an electron-dense structure (Fig. 6, *C*, *D*, and *E*). In ~20% of the cells with a CDC31 signal adjacent

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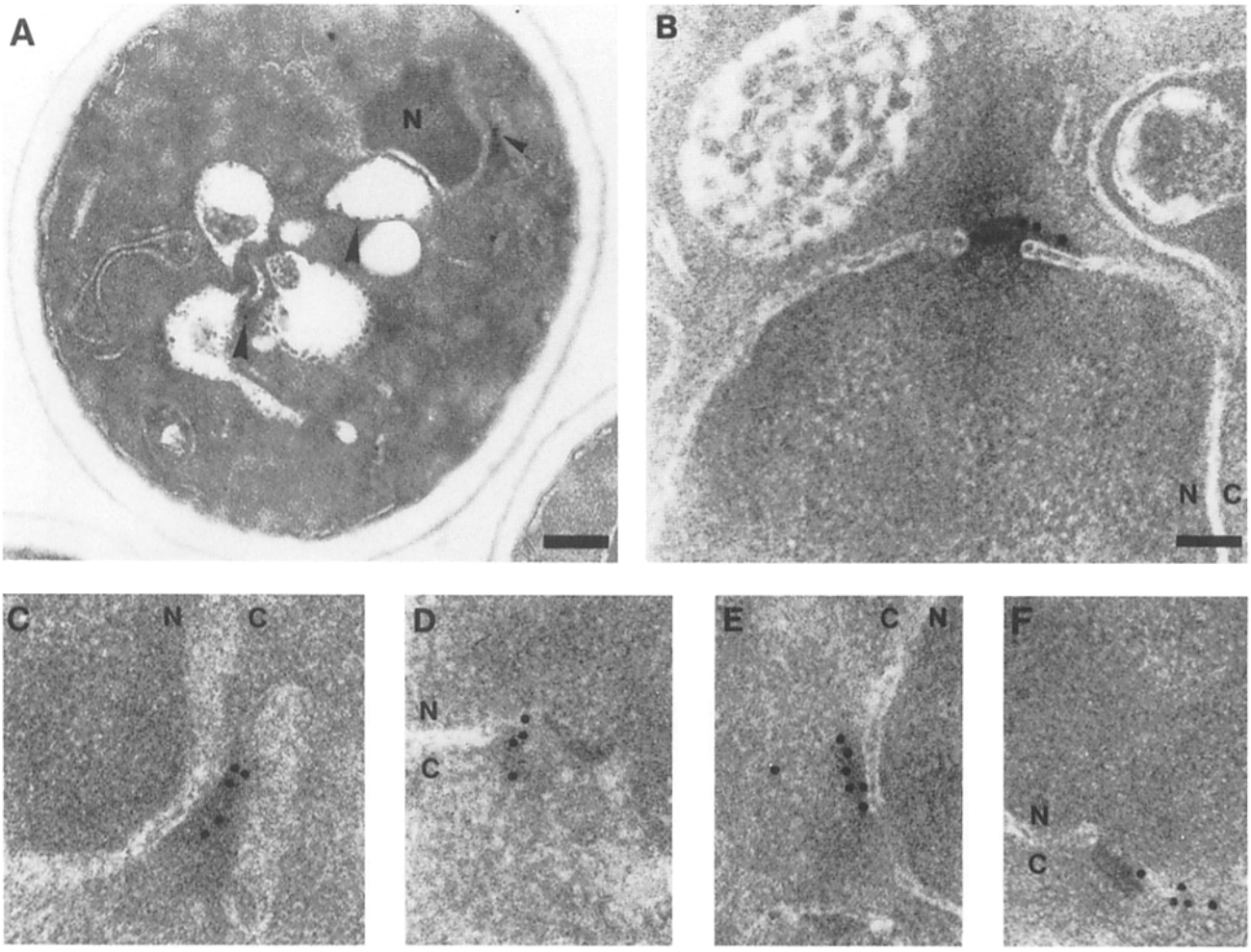

Figure 6. Immunoelectron microscopy of whole yeast cells with anti-CDC31 antibodies (see Materials and Methods). (*A*) Two large arrows indicate the signal obtained with anti-CDC31 antibodies in the cytoplasm. A small arrow indicates a gold particle associated with the nuclear envelope. (*B*) CDC31 associated with a SPB. (*C*) Enlargement of *A*. (*D–F*) CDC31 associated with the nuclear envelope (*D* and *E*) or SPB (*F*). Abbreviations: *N*, nucleus; *C*, cytoplasm. Bars: (*A*) 1.5 μm; (*B*) 0.15 μm. (*C–F*) are the same magnification as *B*.

to the nuclear envelope, CDC31 could be seen in close proximity to a SPB (Fig. 6, *B* and *F*). Fig. 6, *B* and *F* show SPBs that have been sectioned such that the central plaque of the SPB is clearly visible in a discontinuity of the nuclear envelope membrane. Also visible in the micrograph (Fig. 6 *B*) is the SPB half bridge which appears as an electron-dense extension of the central plaque in intimate association with the cytoplasmic face of the nuclear envelope membrane. The outer plaque appeared as a slightly electron-dense layer directed towards the cytoplasmic side of the nuclear envelope membrane (Fig. 6, *B* and *F*). No microtubules associated with the SPBs could be detected. Poor microtubule preservation appears to be a shortcoming of the procedure used for sample preparation (D. Botstein, personal communication). The results of these immunoelectron microscopy experiments were consistent with localization of CDC31 to the half bridge. If the SPB plaques and half bridge are both sectioned in a single cut (see Fig. 8 *A*), both structures will be visible in the electron micrograph (Fig. 6, *B* and *F*). Alternatively, if a cut is made such that the half bridge and not the SPB is sectioned (see Fig. 8 *A*, *thick black square*), then the situation in Fig. 8 *B* will be seen where the half bridge appears as a distinct site, intimately associated with the cytoplasmic face of the nuclear envelope, albeit in apparent isolation of the SPB. We propose that the electron micrographs shown in Fig. 6, *C*, *D*, and *E* were obtained by sections of this type.

CDC31 Associates with the Half Bridge of SPBs

To establish the association of CDC31 with the half bridge of the SPB, immunoelectron EM was performed with enriched SPBs and affinity-purified anti-CDC31 antibodies. The ultra-thin sections showed numerous SPBs of which ~1,000 were inspected more closely. The outer and central plaques of the SPBs and the intermediate line were visible as electron-dense substructures of the SPBs (Fig. 7). The central plaque was interrupted by a less electron-dense layer which we have named the central line. The half bridges were electron dense and extended from the central plaques (Fig. 7, *A–F*). The 20-nm particles seen faintly in the micrographs were Percoll, which was not completely removed by two high speed centrifugation steps following the Percoll density gradient (Rout and Kilmartin, 1990). 2–6 gold particles were associated with ~80% of the sectioned SPBs. These gold

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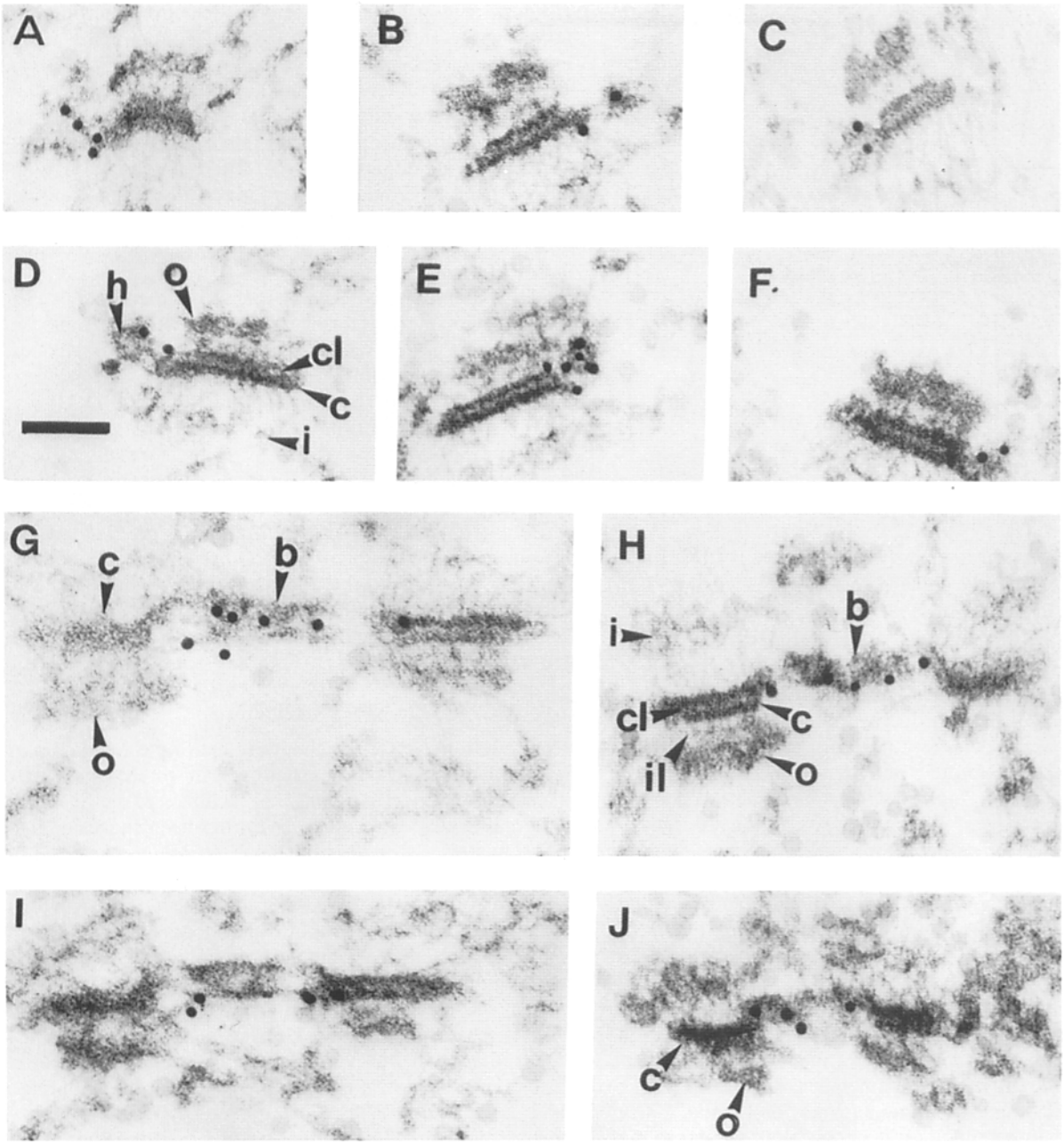

Figure 7. Immunoelectron microscopy of purified SPBs with anti-CDC31 antibodies. (see Materials and Methods). Single SPBs are shown in (*A–F*). (*G–J*) Mother and daughter SPBs connected by the bridge. Abbreviations: *b*, SPB bridge; *c*, central plaque; *cl*, central line; *h*, SPB half bridge; *i*, inner plaque; *il*, intermediate line; *o*, outer plaque. Bar, 81 nm.

particles were localized to the half bridges of the SPBs, adjacent to the central plaques (Fig. 7, *A–F*). No specific SPB staining was obtained with preimmune serum (data not shown).

Late in G1 of the cell cycle, the SPB duplicates and the two SPBs are connected by a bridge formed by interaction of the two SPB half bridges (Byers, 1981*b*). These duplicated, side-by-side SPBs appeared with a frequency of ~1% in our SPB preparation. Representative examples are shown in Fig. 7, *G–J*. The bridge appeared as an electron-dense layer connecting the central plaques of the mother and daughter SPBs. Four to seven gold particles were specifically localized with the center or cytoplasmic side of the SPB bridge indicating a subdivision of this SPB structure.

Discussion

Localization of the CDC31 Protein

Relatively little is known about the molecular structure and cell-cycle regulation of the yeast SPB, despite the fact that the organelle plays a vital role throughout the life cycle of the cell. A number of proteins have been implicated in SPB function, for example, MPS1 and MPS2 (Winey et al., 1991) and NDC1 (Thomas and Botstein, 1986). However, convincing evidence for a structural role has been reported for only four proteins to date; CIK1, KAR1, and the 90- and 110-kD proteins (Page and Snyder, 1992; Vallen et al. 1992*b*; Rout and Kilmartin, 1990). In this paper, we provide the first di-

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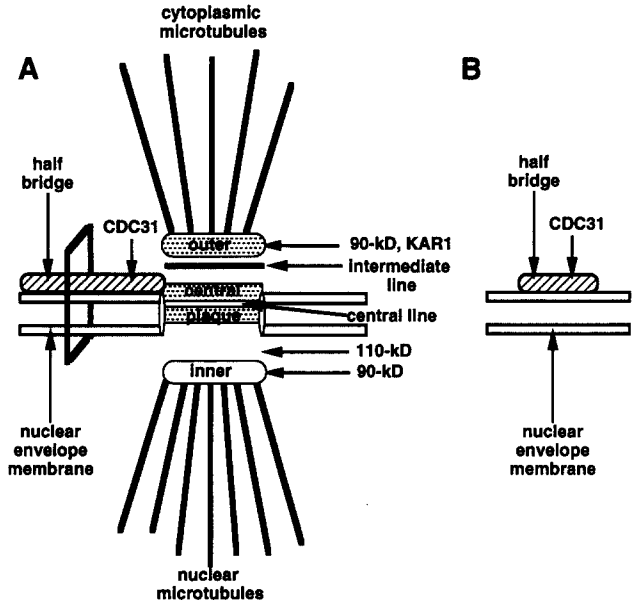

Figure 8. Schematic diagram showing localization of CDC31 relative to that of known SPB components. For a description see text.

rect evidence that CDC31 is a structural component of the SPB. Taken together, four lines of evidence support our conclusion: (*a*) CDC31 cofractionated with enriched preparations of SPBs (Fig. 3); (*b*) double-labeling immunofluorescence experiments demonstrated that CDC31 colocalizes with the 90-kD SPB protein and is closely associated with nuclear microtubules (Fig. 5); (*c*) immunoelectron microscopy of thin sections of yeast cells revealed localization of CDC31 with an electron-dense structure which was immediately adjacent to the SPB plaque (Fig. 6, *B* and *F*); (*d*) immunoelectron microscopy of isolated SPBs established that CDC31 localizes to the half bridge of the single SPB, or to the bridge structure of duplicated but unseparated SPBs (Fig. 7).

Fig. 8 summarizes our results and the data from various laboratories regarding SPB morphology and the localization of SPB components. Inner and outer membranes of the nuclear envelope are fused at the point where the central plaque inserts into the double membrane (Winey and Byers, 1992; Fig. 6 *B*), creating a topological arrangement similar to the insertion of the pore complex into the nuclear envelope (Starr and Hanover, 1990). The central plaque is interrupted by a less electron-dense layer (Fig. 6, *B* and *F*; Fig. 7), which we have named the central line (Fig. 8 *A*). The central line represents a previously undescribed substructure of the SPB. Interestingly, in the eight cases we inspected, only one of the two SPBs connected by the bridge revealed a central line (Fig. 7, *G–J*), and we tentatively suggest that mother and daughter SPB are structurally different at this stage. The mother SPB is most likely to be the one bearing the central line since this substructure was mostly associated with mature SPBs (Fig. 6, *B* and *F*; Fig. 7, *A–F*). A difference in the structure of mother and daughter SPBs has previously been suspected from the selective binding of a LacZ–KAR1 fusion protein to the daughter SPB (Vallen et al., 1992*b*). The half bridge appeared in thin sections of yeast cells as an electron-dense structure immediately adjacent to the SPB plaques and was localized mainly to the cytoplasmic side of the nuclear envelope (Fig. 6, *B* and *E*). Previous electron micrographs, however, revealed the half bridge as electron-dense, membrane-like layers embedded in the nuclear envelope (Byers, 1981*a*). These differences in the appearance of the half bridge most likely reflect the different conditions used to prepare the yeast cells for electron microscopy (Preuss et al., 1991; Byers and Goetsch, 1975). The intermediate line appeared as an electron-dense structure in between the outer and central plaques (Rout and Kilmartin, 1990; Fig. 7). The inner plaque shown in Fig. 8 *A* was only faintly detectable in our electron micrographs (Fig. 7).

CDC31 is the first SPB component to be identified that associates with the half bridge and bridge (Figs. 6 and 7). Hardly any CDC31 was associated with the SPB plaques, suggesting that the subunit composition of the half bridge is different from that of the plaques. Interestingly, CDC31 was mainly associated with the center and cytoplasmic side of the half bridge (Fig. 6 *B* and 7), which makes it likely that the half bridge is itself heterogeneous in structure. Whether the cytoplasmic side of the half bridge is composed entirely of CDC31 or whether additional proteins are involved, has yet to be determined. Other known SPB components are localized to the inner and outer plaques (90 kD; Rout and Kilmartin, 1990), to the outer plaque only (KAR1; Vallen et al., 1992*b*), and to the region in between the central and inner plaques (110 kD; Rout and Kilmartin, 1990).

Role of CDC31 in SPB Duplication

Relatively little is known about the cell-cycle regulation of SPB duplication. Recently, it has been shown that the BIMA protein from *Aspergillus nidulans* localizes to the SPB in a cell cycle-dependent manner, and it has been suggested that BIMA functions at the spindle poles to promote the onset of anaphase (Mirabito and Morris, 1993). BIMA is most highly related in structure to CDC27 of *S. cerevisiae*, which may play a similar role (Mirabito and Morris, 1993; Sikorski et al., 1991). CDC31 is another protein involved in the cell-cycle regulation of SPB function in *S. cerevisiae.* From our studies, it is clear that CDC31 is essential for cell viability (Fig. 1). Disruption of the *CDC31* gene results in a phenotype similar to that of conditional *cdc31* mutants (Schild et al., 1981; Byers, 1981*a*,*b*). Cells arrest uniformly with large buds. Because the arrested cells were at least two times larger than normal, it is apparent that cell division rather than cell growth is affected if levels of CDC31 fall below a critical threshold. Electron microscopic analysis of *cdc31* mutant cells revealed two remarkable features of the SPB. Only one SPB, which was abnormally large and in addition lacked a satellite, was observed (Byers, 1981*a*; A. Spang, unpublished result). Whether the enlarged SPB bears a half bridge or whether the half bridge is an integral part of the enlarged structure, has yet to be determined. The presence of an enlarged SPB in *cdc31* mutant cells suggests that SPB growth but not duplication takes place even in the absence of functional CDC31. The first intermediate structure detectable in SPB duplication is the satellite which forms at the cytoplasmic side of the half bridge (Byers 1981*a*,*b*). The association of CDC31 exactly with this site of the SPB (Figs. 6 and 7) strongly argues for a direct role of CDC31 in SPB duplication, most likely in the formation of the satellite. This conclusion is further supported by the finding that the execution point of CDC31 is coincident with satellite formation

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(Winey et al., 1991). Since no satellite is formed in *cdc31* mutant cells, SPB duplication takes a defective pathway resulting in the formation of a single, deformed SPB.

Mutants of *kar1* (Rose and Fink, 1987) and *cdc31* (Byers, 1981*a*,*b*) reveal a similar phenotype: they arrest as large-budded cells with a single, enlarged SPB lacking a satellite. This finding suggests that the two proteins act in the same pathway in SPB duplication. Experiments with a KAR1–LacZ fusion protein, whose association with SPBs was impaired in *cdc31* cells (Vallen et al., 1992*b*), indicate that KAR1 acts downstream of CDC31. A direct interaction of KAR1 and CDC31 seems unlikely since the two proteins localize to different substructures of the SPB complex (Vallen et al., 1992*b*; Fig. 8).

A direct consequence of the SPB duplication defect may be the cell cycle arrest of *cdc31* mutants. Mutants of *cdc31* enter mitosis with an incomplete, monopolar spindle that is detected by checkpoint controls. Consequently, the mutant cells are uniformly arrested in the cell cycle (Li and Murray, 1991; Hoyt et al., 1991). The homology of CDC31 with calmodulin raises the possibility that CDC31, like calmodulin, is a multifunctional protein. We note that a fraction of the CDC31 signal in immunoelectron microscopy was localized to structures other than the SPB (Fig. 6 *A*). It is worth considering that CDC31 may have functions in addition to its role in SPB duplication. The role of CDC31 in SPB duplication could be the most apparent because it causes cell-cycle arrest.

Ca^{2+} Binding to CDC31

The question remains as to how the CDC31 protein mediates its function in SPB duplication. The immunofluorescence microscopy studies with anti-CDC31 and anti-90-kD antibodies using nuclei from an unsynchronized culture indicated a high degree of colocalization of these two SPB components (Fig. 6), suggesting that a cell cycle–dependent binding and release is an unlikely mechanism for CDC31 function. In this report, we demonstrate that CDC31 binds Ca^{2+}, as predicted from the derived amino acid sequence (Baum et al., 1986), and that this binding is highly specific (Fig. 2). A key step in SPB duplication could be triggered by a conformational change in CDC31 in response to a cell cycle–dependent binding/release of Ca^{2+}. Cell cycle–dependent requirements for calcium are indicated by the transient arrest in G1 of the cell cycle, followed by block at G2/M of the cell cycle if the intracellular calcium stores of yeast cells were depleted (Anraku et al., 1991). SPB duplication seems unaffected by low intracellular calcium concentrations. While this does not exclude a calcium requirement in SPB duplication, it indicates that SPB duplication is not the most calcium-dependent step necessary for cell cycle progression. Unfortunately, to our knowledge, there have been no data published on fluctuations in the cytosolic Ca^{2+} concentration during cell-cycle progression in yeast. This makes it impossible to evaluate whether the intracellular Ca^{2+} concentration changes around the execution point of CDC31.

Taking the homology of CDC31 with calmodulin into account, CDC31 could interact with catalytic proteins such as phosphatase (Liu et al., 1991) or kinase (Antebi and Fink, 1992; Miyakawa et al., 1989), which would transmit the conformational changes of CDC31. Alternatively, CDC31 could function directly as a nucleation site for further SPB components which assemble to form the satellite.

CDC31 Is a Member of the Centrosome-associated, Ca^{2+}-binding Protein Family

Ca^{2+}-binding proteins seem to be universal components of centrosomes and SPBs from a variety of organisms. In *Chlamydomonas reinhardtii* (Huang et al., 1988) and *Tetraselmis striata* (Salisbury et al., 1984), for example, the highly related proteins caltractin and centrin, which share 50% identity with CDC31, are associated with the basal body complex, the functional homologue of the centrosome. Anti-centrin antibodies recognize a 165-kD protein associated with the centrosomes of PtK2 cells (Baron and Salisbury, 1988). Anti-centrin antibodies also decorate the centrosomes of human cultured cells. In immunoblots, these antibodies identify a major centrosomal component which appears as a doublet of 62/64-kD proteins, one of which was found to bind calcium (Moudjou et al., 1991). Interestingly, the nucleation reaction of microtubules was blocked by anti-centrin antibodies, which implies that the 62/64-kD proteins are involved in the nucleation of microtubules (Moudjou et al., 1991). The sequence homology of CDC31 to caltractin (Huang et al., 1988), its calcium-binding properties (Fig. 2), and the association of CDC31 with SPBs (Fig. 3, and 5–7), makes CDC31 a further member of this group of centrosome-associated, calcium-binding proteins. With *CDC31*, it has been shown for the first time that disruption of the gene encoding one of the centrosome-associated, calcium-binding proteins is lethal (Fig. 1). Whether or not the function of these proteins is similar remains to be determined. The localization of CDC31 with the half bridge, which is not involved in microtubule organization, suggests non-identical functions of CDC31 and the 62/64-kD proteins. It is tempting to speculate that control of centrosome and SPB function mediated by a Ca^{2+}-binding protein is an evolutionarily conserved mechanism. Understanding the role played by CDC31 in duplication of the yeast SPB will provide insight into the molecular mechanisms of centrosome function in higher organisms.

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