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An Inhibitor of Yeast Cyclin-Dependent Protein Kinase Plays an Important Role in Ensuring the Genomic Integrity of Daughter Cells

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The gene encoding a 40-kDa protein, previously studied as a substrate and inhibitor of the yeast cyclin-dependent protein kinase, Cdc28, has been cloned. The DNA sequence reveals that p40 is a highly charged protein of 32,187 Da with no significant homology to other proteins. Overexpression of the gene encoding p40, *SIC1*, produces cells with an elongated bud morphology similar to that of cells with depleted levels of the *CLB* gene products, suggesting that p40 acts as an inhibitor of Cdc28-Clb complexes in vivo. A *SIC1* deletion is viable and has highly increased frequencies of broken and lost chromosomes. The deletion strain segregates out many dead cells that are primarily arrested at the G_2 checkpoint in an asymmetric fashion. Only daughters and young mothers display the lethal defect, while experienced mothers appear to grow normally. These results suggest that negative regulation of Cdc28 protein kinase activity by p40 is important for faithful segregation of chromosomes to daughter cells.

Cell division is a highly complex process in which all of the cell's components must be duplicated and accurately segregated to the daughter cells. An elaborate regulatory system has evolved to ensure that synthetic and localization steps occur when and where they are needed and that progression through the cell cycle is responsive to environmental cues. The coordination of these processes appears to be mediated in large part through controls on the activity and localization of the cyclin-dependent protein kinases (Cdks) (reviewed in reference 23). For example, binding of cyclin B to $p34^{cdc2}$ and the subsequent phosphorylation on threonine 167 of $p34^{cdc2}$ produces an active kinase (MPF) that promotes entry into the mitotic phase of the cell cycle. The presence of unreplicated or damaged DNA in the cell, however, promotes additional phosphorylations on $p34^{cdc2}$ (threonine 14 and tyrosine 15) that are inhibitory and prevent entry into mitosis, thus ensuring that mitosis occurs only when the chromosomes have been faithfully duplicated.

The budding yeast *Saccharomyces cerevisiae* has been one of the best model systems for the identification and genetic analysis of the cell cycle. Surprisingly, the best biochemically understood mechanism for the inhibition of cell cycle progression in other eukaryotes—phosphorylation on Tyr-15 of $p34^{cdc2}$ (equivalent to Tyr-19 of Cdc28 of *S. cerevisiae*) in response to incompletely replicated or damaged DNA—does not appear to be operative in *S. cerevisiae* (3, 46). Since *S. cerevisiae* does pause in G_2 as a response to DNA damage (48), there must exist other mechanisms by which cell cycle progression can be modulated. A candidate for this mechanism has become available with the recent discovery of inhibitors of Cdk activity (18, 19, 30, 42, 54). One of these, *CIP1* (also called *WAF1* or p21) is induced by the p53 tumor suppressor/oncogene (13) and may be the important intermediate by which p53 mediates its role as an inhibitor of cellular proliferation in response to DNA damage.

This paper describes the initial characterization of the gene encoding p40, an inhibitor of Cdc28 protein kinase activity which was originally identified as a tight-binding Cdc28 substrate in immunoprecipitates (36). p40 exists in a Cdc28-phosphorylatable form in cycling or arrested G_1-phase cells (31) but not in S-, G_2-, or M-phase cells. In cells arrested by nutritional deprivation or mating pheromone exposure, p40 is not bound to Cdc28 but forms a tight complex when mixed with active Cdc28 derived from S-, G_2-, or M-phase cells (31, 53). We have used these binding and phosphorylation properties to purify p40 from stationary-phase yeast cells and have shown that the purified protein inhibits the phosphorylation of histone H1 by Cdc28 in vitro (30). As this represents a novel mechanism by which Cdc28 may be regulated, we have undertaken a genetic analysis of the gene encoding p40. Here we show that overproduction of p40 generates a phenotype consistent with inhibition of Cdc28 in vivo, that it has an important role in ensuring genomic integrity, and that this role has a pronounced mother-daughter asymmetry. We propose that p40 is a negative regulator of Cdc28 that acts in response to a signal from a post-START checkpoint.

MATERIALS AND METHODS

Protein methods. The purified p40 preparation used for peptide sequencing was from the same preparation as that used by Mendenhall (30). Aliquots of p40 containing 35 μg of protein were digested with 1.4 μg of chymotrypsin or 0.7 μg of *Staphylococcus* V8 protease for 24 h at 37°C as described by Stone et al. (48) and Allen (2). The digestion products were purified by reverse-phase high-performance liquid chromatography (HPLC) on a Phenyl μBondapak column with a gradient of 0.1% trifluoroacetic acid (TFA) to 44% acetonitrile–0.025% TFA (12). The peptides were sequenced on an Applied Biosystems 477A pulsed liquid-phase protein sequencer. Phosphorylation assays of p40 were done as described by Mendenhall (30), using a mouse monoclonal antibody specific for p40

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to immobilize p40 to microtiter dishes. Protein extracts used for the phosphorylation assays were prepared by vortexing cells and an equal volume of 50 mM Tris (pH 7.5), 0.008% Triton X-100, 1 mM dithiothreitol, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 μg of aprotinin per ml, and 0.125 μg of phenylmethylsulfonyl fluoride per ml with glass beads. The phosphorylation reactions were carried out in 10 mM Tris (pH 7.5), 7.5 mM $MgCl_2$, 0.5 mM $ZnSO_4$, and 250 μCi of [γ-^{32}P]ATP (ICN; >3,000 Ci/mmol) per ml.

DNA methods. The *SIC1* gene was cloned by screening a yeast genomic DNA λDASH library from Stratagene with an 18,432-fold degenerate mixture of oligonucleotides with the pattern GARAAYCCNGAYATHGARGAYGTNATH ACNTA (where R = A or G; Y = T or C; H = A, T, or C; and N = A, G, T, or C) end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The probe was hybridized to the filters in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–0.25% nonfat milk at 60°C overnight and then washed five times quickly at room temperature, twice for 5 min each wash at room temperature, once for 3 min at 60°C in 6× SSC–0.1% Sarkosyl, and then once more for 30 s at room temperature in 2× SSC.

The DNA was sequenced directly from the purified λ phage or from plasmid subclones with a kit based on a thermal cycling method from Bethesda Research Laboratories. Oligonucleotide primers were synthesized so that both strands of the coding region were sequenced. The EMBL and GenBank data bases were searched for sequences similar to *SIC1* with the NCBI BLASTN and FASTDB programs. A specialized data base compiled by Mark Goebl (Indiana University School of Medicine) was also searched. Physical mapping of the *SIC1* gene was carried out by screening the Riles-Olson ordered array of yeast genomic clones with an internal fragment of *SIC1* generated by PCR, using the protocols supplied by L. Riles (Washington University).

The deletion-disruption mutation was constructed by first subcloning the *SIC1* gene from one of the λDASH clones into pGEM-4Z on an *Eco*RI-*Hin*dIII fragment to create pMDMb143. An internal *Asp* 718-*Nde*I fragment of *SIC1* was replaced with a *Bam*HI fragment from YDp-H (6) containing the yeast *HIS3* gene. The resulting plasmid, pTNb18, was then cut with *Eco*RI and *Nco*I (5′ and 3′ to translated region, respectively) and transformed into the diploid yeast strain YPH274 (*MAT***a**/*MAT*α homozygous *ade2-101*^{o} *his3-Δ200 leu2-Δ1 lys2-801*^{a} *trp1-Δ1 ura3-52*) (45). YPH274 was formed from two isogenic strains, so all haploids products should have identical genotypes. The pTNb18 transformant of YPH274 was then dissected to produce the sister spores TTN1-3A (*MAT*α *sic1-Δ1*::*HIS3*), TTN1-3B (*MAT***a** *sic1-Δ1*::*HIS3*), TTN1-3C (*MAT***a** *SIC1*$^+$), and TTN1-3D (*MAT*α *SIC1*$^+$) used for all subsequent analysis.

The galactose-inducible allele was constructed by first creating a *Bam*HI site at the 5′ end of the *SIC1* gene by PCR with a 5′ primer that incorporated a *Bam*HI recognition sequence. The PCR fragment was then ligated to an *Eco*RI-*Bam*HI fragment from pBM150 containing the *GAL1* promoter (26), and the resulting construct was cloned into the set of YIp, YCp, and YEp vectors described by Gietz and Sugino (14). The yeast strain TTN1-3C was transformed with pMDMb151 (YCp*GAL1*→*SIC1 URA3*) to create the p40 overproducer. For the integrated version, pMDMb169 (YIp*GAL1*→*SIC1 URA3*) was linearized with *Eco*RV to direct integration into the *URA3* locus of EY957 (*MAT***a** *bar1Δ can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 GAL*$^+$; gift of E. Elion, Harvard Medical School).

Other methods. Plating efficiency was measured by dividing the number of yeast colonies appearing on plates by the number expected from hemacytometer counts of the cells in liquid culture. Staining of cells with propidium iodide for flow cytometry was done as described by Hutter and Eipel (22) except that after staining, the cells were not washed but were diluted to give a final concentration of less than 10^6 cells per ml. Flow cytometry was carried out on a Becton Dickinson FACS IV instrument. A total of 50,000 cells were observed for each sample. Chromosomal breakage and loss measurements were determined by the quantitative mass mating procedure described by Sprague (47), using the tester MDMy619 (*MAT*α *ade6 his4-580*^{a} *leu2 thr4 trp1-1*^{a} *tyr1*^{o}). UV sensitivity was determined with a 254-nm Westinghouse germicidal bulb. The dose was determined with a UVX digital radiometer. All operations were performed under yellow light to avoid photorepair of thymine dimers. Gamma ray sensitivity was determined by exposing the cells with a Mark I ^{137}Cs irradiator in liquid culture and plating cell dilutions onto YPD (1% yeast extract, 2% Bacto Peptone, 2% dextrose) plates to determine the number of survivors. All other techniques related to handling of bacteria, phage, plasmids, and DNA are as described by Sambrook et al. (40). General yeast techniques were described by Rose et al. (39) and Guthrie and Fink (17). Sorbitol (1 M) was included in solid media used for dissection and pedigree analysis to improve viability.

Nucleotide sequence accession number. The nucleotide sequence data have been deposited in the EMBL, GenBank, and DDBJ data bases under the accession number U01300.

RESULTS

Cloning the p40 gene. The gene encoding p40 was cloned by screening a λ library of yeast genomic DNAs with a degenerate mixture of all oligonucleotides capable of encoding a sequenced peptide derived from p40. To do this, the p40 polypeptide was purified as previously described (30). An initial attempt to sequence the amino terminus was unsuccessful. This was most likely due to the presence of an N-terminal blocking group, a common feature of proteins obtained from eukaryotic sources. A fresh preparation of the purified protein was then subjected to chymotryptic cleavage by the method of Stone et al. (48). To reduce potential disulfide bonds, a 17.6 μM solution of p40 was treated with 5 mM dithiothreitol in 8 M urea–400 mM NH_4HCO_3 prior to cleavage. Immediately upon the addition of dithiothreitol, the solution turned noticeably orange. Proteins eluting immediately before and after p40 in the last step of the purification procedure (reverse-phase chromatography on Pharmacia ProRPC) remained colorless when similarly treated. The p40 solution became colorless when heated to 50°C, but the color returned as the solution cooled to room temperature. Treatment with 10 mM iodoacetamide permanently destroyed the color. These results indicate that an oxidization-sensitive chromophore is bound to the p40 polypeptide, perhaps covalently. The nature or function of this chromophore has not been further investigated.

The chymotryptic fragments of p40 were separated by HPLC, and the amino acid sequences of two polypeptides were determined (Table 1, peptides I and II). An additional polypeptide sequence of a fragment obtained from *Staphylococcus* V8 protease cleavage was also determined (Table 1, peptide III). A mixture of all oligonucleotides capable of encoding the last 11 amino acids of polypeptide I was synthesized and used as a hybridization probe to screen a λ library of *S. cerevisiae* genomic sequences. Of 40,000 plaques screened, 12 gave positive signals with this probe. Preliminary restriction maps of 10 of the phage DNAs indicated that two were identical. The maps of the others differed but shared an

TABLE 1. Sequences of polypeptide fragments of p40 and comparison to sequences obtained from the *SIC1* gene

Source[a]	Amino acid sequence
Peptide I	LRENPDIEDVITY
DNA (aa 238–250)	LRENPDIEDVITY
Peptide II	ELAKNEF
DNA (aa 182–188)	ELAKNWN
Peptide III	KRRLT?EE?RRF?P?ALFQSRDQE
DNA (aa 260–283)	KRRLTDEEKRRFKPKALFQSRDQE

[a] aa, amino acids.

identical region surrounding the site of probe hybridization. The DNA sequence of this region was determined. Analysis of this sequence identified an open reading frame capable of encoding a 284-amino-acid protein with a predicted molecular mass of 32,187 Da (Fig. 1). The predicted polypeptide contains close or perfect sequence matches to polypeptides I, II, and III (Table 1). There was no significant sequence similarity between the p40 protein sequence and sequences in the GenBank, EMBL, SwissProt, PIR, and dbest (expressed sequence tags) data bases, sequences in the specialized data base compiled by Mark Goebl (15), or the sequences of other recently isolated Cdk inhibitors (9, 18, 19, 42, 54). The p40 coding sequence is identical to *SDB25* (11), a high-copy-number suppressor of *DBF2* (25). The 5′ noncoding region was identical to the noncoding region 3′ to the *BOS1* gene (44), indicating that these genes are adjacent to each other in the yeast genome. The initiation codon of the gene encoding p40 is 260 bp from the termination codon of *BOS1*. The p40 gene hybridized to λ clones 3688, 4673, and 6637 of the Riles-Olson collection, indicating that it mapped between *gal2* and *spt8* on the right arm of chromosome XII (37). Given the in vitro properties of p40 and the phenotypic analysis described below, we have named the gene *SIC1* for substrate/subunit/inhibitor of cyclin-dependent protein kinase.

Overproduction of p40 produces a transient arrest of nuclear but not budding cycles. Since purified p40 inhibited the phosphorylation of histone H1 and of a peptide substrate by the Cdc28 protein kinase (30), we sought to test whether it had a similar action in vivo by observing the effects of p40 overproduction on the yeast cell cycle. The entire *SIC1* open reading frame was fused to the inducible *GAL1* promoter on a plasmid vector containing a centromere and an autonomously replicating sequence. A yeast strain transformed with this construct was grown in a selective medium containing sucrose (which neither represses nor induces the *GAL1* promoter) and then shifted to selective medium containing galactose. Roughly 10- to 100-fold-greater amounts of Cdc28-phosphorylatable p40 were detected in extracts from the galactose-induced cells relative to that in the untransformed controls after 6 h of induction (Fig. 2).

Between 10 and 25% of the cells in the induced culture became highly elongated (Fig. 3) and contained a single nucleus, but the other cells retained a normal appearance and continued to divide. The elongated forms resembled morphologies seen when hyperactive Cdc28-Cln complexes are produced or when Cdc28-Clb activity is reduced (27). After prolonged periods (24 h) of p40 production, forms in which the mother cell had two or more elongated buds or which possessed very long tubular buds with periodic constrictions, but which were still uninucleate, were seen. During this period, normal-looking cells increased in number and became the dominant species with continuous culturing. A stable p40 overexpressor, constructed by integrating the *GAL1p→SIC1* construct into the yeast genome and a derivative expressed from the *ADH1* promoter (and which also contained a nuclear localization sequence), had virtually identical morphologies. The fraction of cells possessing the tubular morphology varied from experiment to experiment and appears to be a function of the nutritional status of the culture, since the higher frequencies appeared in cultures that were grown at low (<0.5%) concentrations of sugar or that were shifted from stationary-phase cultures. The G_2/M cells were almost completely eliminated in flow cytometric analyses of the *GAL1p→SIC1* cells compared with controls. However, separate analysis of only the largest cells in the population, which should be enriched for the cells with the tubular morphology, indicated that this subpopulation possessed G_2/M and higher levels of DNA (data not shown). Additional studies will be needed to ascertain the effect of *SIC1* overexpression on DNA synthesis, but we interpret these results to indicate that overexpression of *SIC1* can inhibit the cell cycle progression of a subset of the cells in a population, most likely by inhibiting the activity of Cdc28-Clb complexes.

Loss of p40 results in partial mitotic arrest. As a first step towards determining the in vivo function of p40, we constructed a deletion-disruption mutation of the *SIC1* gene in a diploid yeast strain. The region encoding amino acids 15 to 248 was replaced with the yeast *HIS3* gene flanked by translation termination modules from YDpH (6) to create the *sic1-Δ1::HIS3* allele (Δ*sic1*) in vitro. This plasmid construct was

```
        10         20         30         40         50         60
MTPSTPPRSRGTRYLAQPSGNTSSSALMQGQKTPQKPSQNLVPVTPSTTKSFKNAPLLAP

        70         80         90        100        110        120
PNSNMGMTSPFNGLTSPQRSPFPKSSVKRTLFQFESHDNGTVREEQEPLGRVNRILFPTQ

       130        140        150        160        170        180
QNVDIDAAEEEEEGEVLLPPSRPTSARQLHLSLERDEFDQTHRKKIIKDVPGTPSDKVIT

       190        200        210        220        230        240
FELAKNWNNNSPKNDARSQESEDEEDIIINPVRVGKNPFASDELVTQEIRNERKRAMLRE

       250        260        270        280
NPDIEDVITYVNKKGEVVEKRRLTDEEKRRFKPKALFQSRDQEH
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FIG. 1. Amino acid sequence of the p40 protein predicted from the *SIC1* DNA sequence.

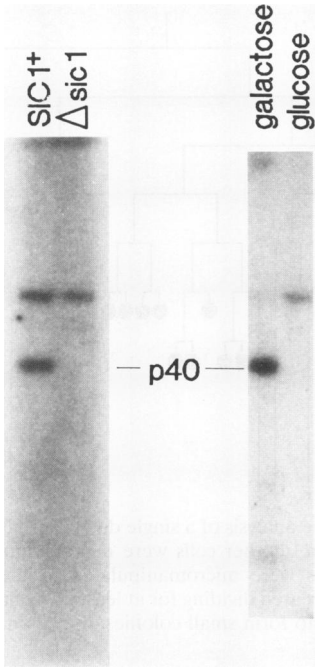


FIG. 2. Autoradiograph of CDC28 phosphorylation assays of Δ*sic1* and *GAL1p→SIC1* extracts. The presence of p40 was detected by adding crude yeast lysates to microtiter dishes previously coated with a mouse monoclonal antibody to p40 and blocked with Tween 20. After a 1-h incubation on ice, the plates were washed and a partially purified extract containing the Cdc28 protein kinase was added. The plates were incubated for an additional hour on ice and then washed again. [γ-^{32}P]ATP was added, and the plates were incubated at room temperature for 30 min. The reaction was stopped with SDS-polyacrylamide sample buffer, boiled, and loaded onto an SDS-polyacrylamide gel. (Left panel) Assays of 2 A_{280} units of extracts from TTN1-3D (*SIC1*$^+$) and TTN1-3B (Δ*sic1*); (right panel) assays of 0.03 A_{280} unit of extracts from yeast strain MDMy550 (*GAL1p→SIC1*) grown in 2% galactose or 2% glucose. The position of phosphorylated p40 is indicated. The identity of the higher-molecular-weight phosphoprotein is unknown, but its presence is contributed by both the crude yeast extracts and the CDC28 preparation. Its phosphorylation is dependent upon CDC28 but is not dependent upon the addition of anti-p40 antibody.

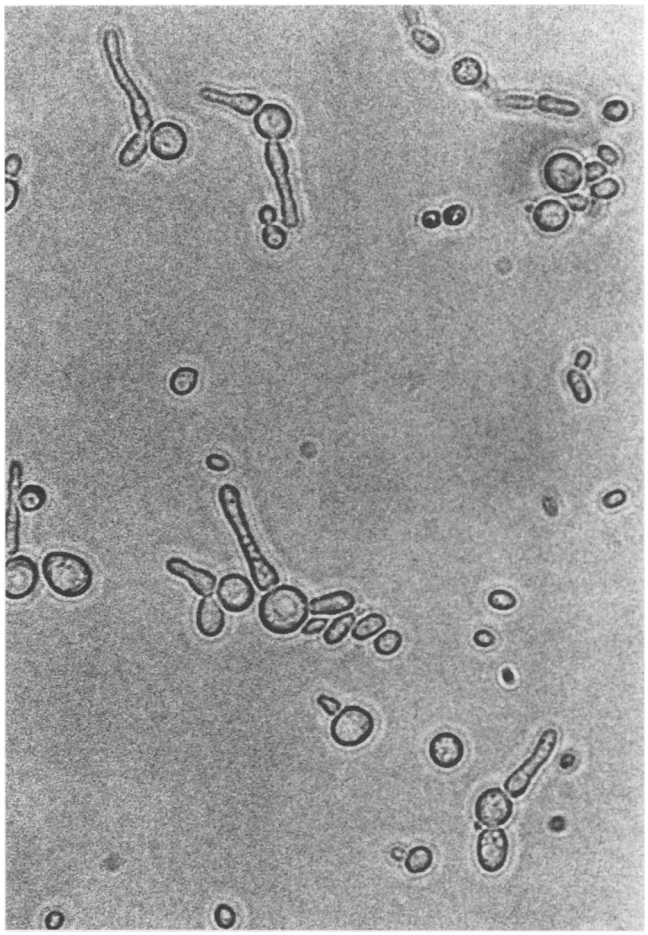


FIG. 3. Effects on cellular morphology of overexpression of *SIC1* from the *GAL1* promoter.

then linearized with restriction enzymes that cut 5′ and 3′ to the remaining *SIC1* sequences and was used to transform a *his3*$^-$ diploid to histidine prototrophy. Southern blot analysis indicated that one of the two alleles of *SIC1* had the restriction pattern expected for the disrupted allele (data not shown). The diploid was sporulated and dissected. Viability was excellent, indicating that *SIC1* was not an essential gene, but Δ*sic1* cultures grew noticeably more slowly. The doubling time of 20 Δ*sic1* cultures derived from independent spores averaged 4.1 h (standard deviation, 0.8 h), which is significantly longer than the average 2.7-h doubling time (standard deviation, 0.4 h) of the *SIC1*$^+$ sister spores. Protein extracts from the Δ*sic1* strain were assayed for p40 by phosphorylation with the Cdc28 protein kinase (Fig. 2). No p40 protein was detected, indicating that the *SIC1* gene had been deleted and that it did, in fact, encode the p40 protein.

Cultures of Δ*sic1* cells had a high frequency of large-budded cells (Fig. 4) with a single, unelongated nucleus at the neck between the mother and daughter (21% of the population versus 5% for wild-type cultures). The Δ*sic1* cells with this morphology were frequently highly enlarged. Since yeast cells usually continue to add mass when the cell division cycle is halted, the abnormal size of the large-budded cells suggested that the Δ*sic1* cells had difficulty completing a post-START event.

To examine this phenotype in more detail, the division of individual cells was observed through several generations. At each division, the mother was separated from the daughter by micromanipulation. Ninety-eight mitoses in three independent pedigrees were examined (one such pedigree is shown in Fig. 5). In agreement with the microscopic observations of mass cultures, we observed 48 cells which formed a large bud but never divided again. This indicates that the defect in the Δ*sic1* strain did not simply delay mitosis but prevented cell division from occurring in a subpopulation of the culture. Several cases in which cells with this morphology exhibited prolonged division delays (an 8-h delay was observed for one cell) but eventually went on to divide and form colonies were observed. At a lower frequency, other terminal morphologies (seven cells with no buds, two cells with small buds, or one cell with a narrow, elongated bud) were also observed. Division defects did not appear randomly. Cells that had successfully produced two daughters (old mothers) never exhibited a mitotic defect in these pedigrees. When a mother cell died (24 cases), it always died within the first or second generation after its birth (young mothers), and all its descendants also died. This does not indicate that old mothers are normal or even immortal. In

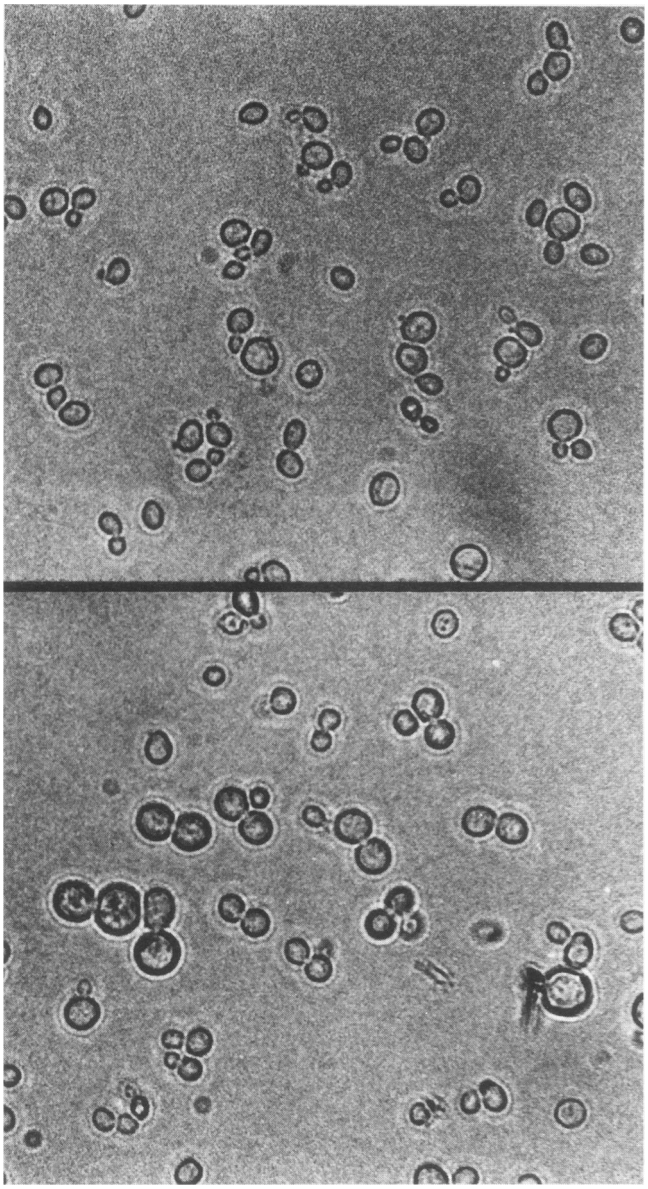


FIG. 4. Comparison of wild-type (top) and Δ*sic1* (bottom) cellular morphologies.

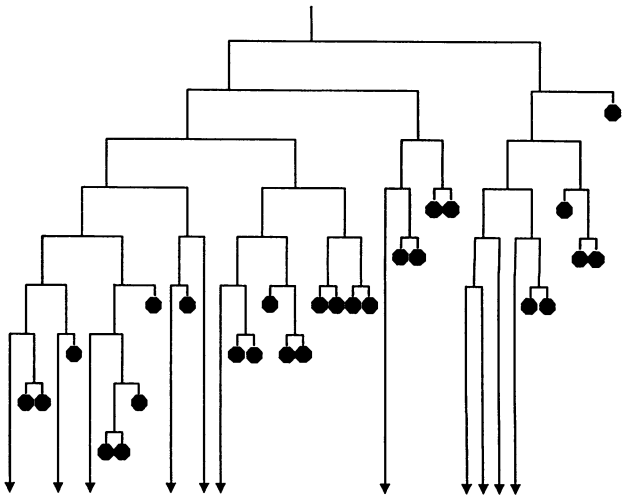


FIG. 5. Pedigree analysis of a single dividing TTN1-3B (Δ*sic1*) cell. After each division, mother cells were micromanipulated to the left and daughter cells were micromanipulated to the right. Octagons indicate cells that ceased dividing for at least 24 h; arrowheads indicate cells that went on to form small colonies after 24 h.

more extensive examinations, Pinswasdi and Jazwinski (34) have confirmed the asymmetric pattern of lethality in Δ*sic1* mitoses but have also found that the old mothers have a significantly reduced life span (24). The frequent appearance of nondividing cells had a measurable effect on plating efficiency. Only 41% of the Δ*sic1* cells in an exponential-phase culture could form colonies when plated compared with 71% of isogenic *SIC1*$^+$ cells.

Flow cytometry provided additional evidence that the Δ*sic1* strain was defective in the initiation of mitosis (Fig. 6). Only a small fraction (~9%) of a Δ*sic1*/Δ*sic1* diploid culture had the 2C DNA content indicative of G_1 phase compared with that (33%) in a *SIC1*$^+$ culture. There was a concomitant increase in the fraction of cells with the 4C DNA content indicative of cells in G_2/M (70% for Δ*sic1* versus 56% for *SIC1*$^+$). In addition, 8% of the cells in the Δ*sic1* culture had a DNA content apparently greater than 4C compared with less than 1% of the cells in the wild-type culture. As expected from the microscopic observations, analysis of the forward light scatter confirmed the existence of a large population of enlarged cells. Almost all of the enlarged cells had a 4C or greater DNA content. There also appears to be an increased population of small cells with 4C DNA content. It is not clear whether this population represents cells that have initiated S phase early, is due to the presence of tetraploid cells in the population, or is just debris resulting from the high rates of cell death in the culture.

The difficulty in mitotic initiation and the presence of cells with increased DNA contents suggested that Δ*sic1* cells might be experiencing high chromosome loss rates as well. To test this, we exploited the ability of cells that had lost the mating type locus (*mat*^{0} cells) to mate as if they were *MAT***a** (28, 29) to measure the frequency at which chromosome III was lost. Quantitative mass matings between the mutant TTN1-3A (*MAT*α *ade2 his3* Δ*sic1 ura3-52*) and the control TTN1-3D (*MAT*α *ade2 his3 ura3-52*) with the tester MDMy619 (*MAT*α *ade6 his4 thr4 tyr1*) were carried out and plated onto a defined medium lacking adenine, tyrosine, and uracil to select for diploids. The frequency of "illegal" matings between the mutant and the tester was 92 times that of the isogenic wild type and tester (Table 2). To distinguish chromosomal loss events from other mechanisms that would allow *MAT*α × *MAT*α matings, we ascertained whether the diploids could grow on a medium lacking histidine or threonine. The *MAT* locus and the *his4* gene are on opposite arms of chromosome III, while the *thr4* locus is distal to *MAT* on the right arm (Fig. 7), so illegal matings due to loss of chromosome III in TTN1-3A or TTN1-3D cells should be auxotrophic for histidine and threonine. Twenty-six percent of the illegal maters fell into this class at a frequency that was 120 times that of the wild type (Table 2), but the largest class (65%) of illegal matings between the Δ*sic1* strain and the tester were Thr$^-$ His$^+$. This class most likely results from the breakage of chromosome III between the *MAT* locus and the centromere and the subsequent loss of the acentromeric fragment. The frequency of this event was elevated 1,200-fold relative to the wild type.

Reversion to auxotrophy of several missense and nonsense

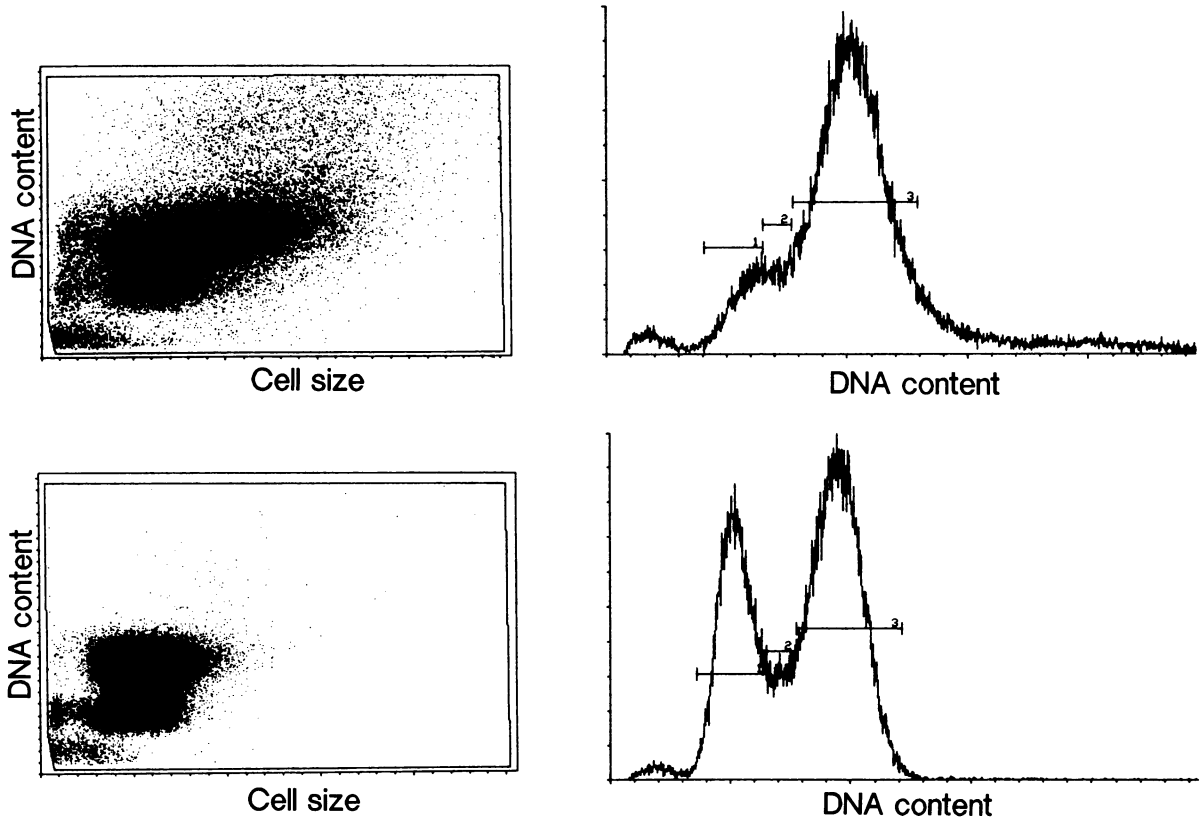


FIG. 6. Flow cytometric analysis of the Δ*sic1* mutant diploids. (Top) MDMy570 (Δ*sic1*/Δ*sic1*); (bottom) MDMy567 (*SIC1*$^+$/*SIC1*$^+$); (left) dot plots of DNA content (propidium iodide fluorescence) versus cell size (forward light scatter); (right) DNA content histograms (graphical representation of data taken from the dot plot in the left-hand panels). Horizontal bars labeled 1, 2, and 3 indicate distribution of cells in G_1, S, and G_2/M, respectively.

mutations (*ura3-1*, *trp1-1*^{a}, and *ade2-101*^{o}) was unaltered in the Δ*sic1* strain (data not shown), indicating that the spontaneous point mutation rate was not increased.

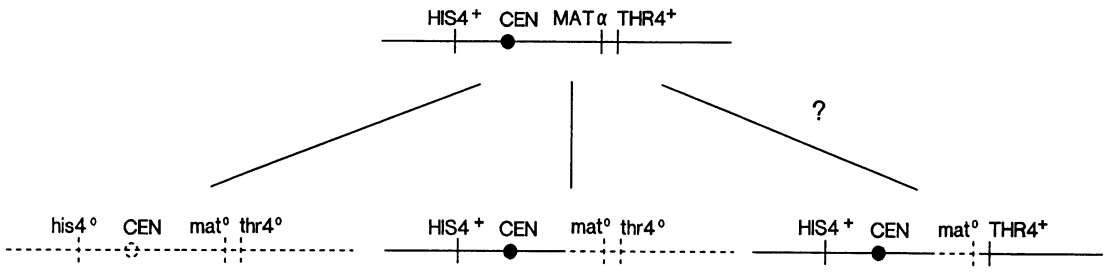
A checkpoint function for p40? The high rate of chromosomal damage observed in the Δ*sic1* strain is reminiscent of the effects of the mitotic catastrophe mutants of *Schizosaccharomyces pombe* (16). Mitotic catastrophe results from premature entry into mitosis due to a loss of negative controls on the p34^{cdc2} protein kinase. Cultures undergoing mitotic catastrophe have high rates of cell death with the appearance of cells with fragmented nuclei and septa cutting through nuclei. This interpretation of the Δ*sic1* phenotype is consistent with the in vitro and probable in vivo function of p40 as an inhibitor of the Cdc28 protein kinase. Such negative controls form part of a system of checkpoints (20) that monitor the successful completion of key cell cycle events and coordinate the timing of these events.

To determine whether *SIC1* is involved in a previously studied checkpoint, we examined the effects of treatments that influence the viability of known checkpoint mutations on the Δ*sic1* strain (21, 28, 52). Wild-type and mutant cells had identical sensitivities to UV irradiation (data not shown). The mutant showed a modest resistance to gamma radiation (data not shown) that was most likely due to the increased fraction of G_2-phase cells in the Δ*sic1* strain relative to that in the wild type (7). Prolonged (7.5-h) arrest in the DNA synthesis inhibitor hydroxyurea (200 mM) had no effect on the plating efficiency. The plating efficiency of the Δ*sic1* strain decreased steadily from 34 to 19% during a 6-h arrest in the microtubule inhibitor nocodazole (10 μg/ml), but the wild-type control also decreased from 85 to 27% during the same interval. Attempts were made to suppress the Δ*sic1* plating defect by growth in sublethal concentrations of hydroxyurea or nocodazole. No changes in plating efficiencies were seen when the cells were

TABLE 2. Frequencies of illegal *MAT*α Δ*sic1* by *MAT*α *SIC1*$^+$ matings[a]

Parents	Frequency		
	Illegal matings	Chromosome loss	Chromosome breakage
Δ*sic1* × tester	2.3×10^{-5} (1.4×10^{-5})	6.8×10^{-6} (6.7×10^{-6})	1.4×10^{-5} (8.2×10^{-6})
SIC1$^+$ × tester	2.5×10^{-7} (3.0×10^{-7})	5.7×10^{-8} (6.2×10^{-8})	1.2×10^{-8} (2.4×10^{-8})
Ratio of Δ*sic1* to *SIC1*$^+$	92	120	1,200

[a] The frequencies presented are the averages of 10 independent experiments with the mutant and 9 independent experiments with the wild type. The standard deviations are given in parentheses.



	Thr⁻ His⁻	Thr⁻ His⁺	Thr⁺ His⁺
Δ sic1	26% (223)	65% (562)	5% (45)
SIC1⁺	43% (17)	7.5% (3)	50% (20)

FIG. 7. Chromosomal breakage and loss in Δ*sic1* mutants. The arrangement of the chromosome III markers of the Δ*sic1* and *SIC1*⁺ strains—*HIS4*, *MAT*α, and *THR4*—relative to the centromere (CEN) are indicated at the top of the figure. These strains were mixed with a *his4*⁻ *MAT*α *thr4*⁻ tester strain, and rare mating events were selected by complementation of additional auxotrophic markers on other chromosomes. The rare illegal maters were replica plated to media lacking either histidine or threonine, and the colonies were classified by their growth properties on these plates. The percentage distributions for the mutant (Δ*sic1*) and the wild type (*SIC1*⁺) are given at the bottom of the figure. The actual number of colonies in each class is given in parentheses. The interpretation of each class is indicated diagrammatically in the central part of the figure. Dotted lines indicate portions of chromosome III that would be lost when generating the different illegal mating classes. The Thr⁻ His⁺ class could also contain events that deleted an internal portion of chromosome III containing *MAT* and *THR4*, while the Thr⁺ His⁺ class would also include other events that allow rare mating to occur such as *MAT* point mutations and switches to the opposite mating type, etc. The Δ*sic1* mutant also produced 31 Thr⁻ colonies that formed His⁺ and His⁻ sectors and 4 colonies that were His⁺ and formed Thr⁺ and Thr⁻ sectors that are not indicated in the figure. No Thr⁺ His⁻ colonies were observed.

treated with 1 to 5 μg of nocodazole per ml for over five doubling times, although doubling times increased to over 12 h at the higher concentrations. The hydroxyurea treatments (50 and 75 mM) were equivocal, as they had no effect on plating efficiency of the Δ*sic1* strain but that of the wild-type control dropped to 32%. This could be interpreted either as having no effect on the Δ*sic1* strain or as resulting from a balance of lethal and suppressive effects. These results suggest that, if *SIC1* is needed for a checkpoint function, it is not involved in the response to DNA damage, unreplicated DNA, or spindle formation failure.

Since p40 is most easily observed in G_1-phase cells (31), we examined the possibility that it is involved in processes known to cause G_1 arrest. The Δ*sic1* strain was not significantly different from the wild type with respect to mating proficiency (in both Δ*sic1* × wild type and Δ*sic1* × Δ*sic1* matings), arrest due to nitrogen starvation, or the acquisition of thermotolerance (data not shown).

DISCUSSION

The structure of the p40 protein. Biochemical analysis of p40 has revealed several unusual features that would not have been predicted from genetic analysis alone. The most striking of these is the apparent existence of an oxidation-sensitive chromophore that was observed upon the addition of dithiothreitol to a concentrated solution of the purified protein. The loss of color upon heating indicated that the tertiary structure of the protein is an important determinant of the chromophore's spectral properties. The rapid return of color upon cooling implies that the chromophore is probably covalently attached to the p40 polypeptide and that the tertiary structure is resistant to permanent denaturation. This is consistent with the observation that the CDC28 binding and substrate properties of p40 are resistant to 5-min incubations in boiling water (personal observations). An additional curious feature is the complete lack of cysteine residues in the encoded polypeptide, since colored proteins often contain metal atoms liganded to cysteine.

The microsequencing of peptide III failed to identify four residues (indicated by question marks in Table 1). The first of these was an aspartate that was masked by coelution with a system contaminant. The other three, all lysines (K-268, K-272, and K-274), were not detected. Instead, a novel peak eluting prior to a diphenylthiourea standard on the amino acid analyzer was found at each of these lysine positions. This apparent, unknown lysine derivative eluted well after mono-, di-, and trimethyllysine standards. Its identity has yet to be established. Another unusual feature of peptide III was the failure of *Staphylococcus* V8 protease to cleave at the pair of glutamate residues (E-266 and E-267) in the middle of the peptide, suggesting that this region of the protein might have a constrained secondary structure. It is not known whether these properties of peptide III are related to the chromophore.

The *SIC1* DNA sequence predicts that p40 is very hydrophilic. Charged residues compose 31% of the amino acids and are concentrated in the carboxy-terminal half of the protein. There are two very acidic stretches (DIDAAEEEEEGE from amino acid 124 to 135 and ESEDEED from amino acid 200 to 206), but the protein is basic overall. This composition may explain the discrepancy between the observed molecular mass for p40 on SDS-polyacrylamide gels (40 kDa) and that predicted from the sequence (32 kDa), as highly charged proteins bind SDS poorly. There is a notable abundance of proline residues (9.5%). Nine sites match the minimal $p34^{cdc2}$ phosphorylation consensus sequence (S/T-P), and three of these are particularly favored by having positively charged residues nearby. The consensus substrate "rules" may not apply to p40, however, because of its unusual ability to bind tightly to the Cdc28-Clb complex. Three of the potential $p34^{cdc2}$ phosphorylation sites also match the consensus for mitogen-activated protein kinase (P-X-S/T-P).

The codon bias index is 0.017 (5), placing *SIC1* among the 10% least-biased genes (43). This is indicative of a poorly

expressed gene and was expected from the low level of p40 found in yeast cells (30). The predicted p40 protein also contains three PEST regions: amino acids 37 to 49 (PEST score −2.0), 115 to 141 (−2.1), and 198 to 212 (+2.0). PEST regions, rich in proline, glutamate, serine, threonine, and aspartate and bounded by positively charged residues, are often found in unstable proteins (38). The range of PEST scores found in p40 is similar to that found in known short-lived proteins such as the p53 tumor suppressor, the oncogene v-*myb*, and the heat shock protein HSP70. The presence of these PEST regions may, in part, explain the rapid loss of assayable p40 in crude yeast cell extracts (30) and play a role in the inactivation of p40 inhibition of Cdc28 in vivo.

The function of p40. Prior studies of p40 have revealed three distinct, but related, activities involving the Cdc28 protein kinase in vitro—binding of p40 to Cdc28, phosphorylation of p40 by Cdc28 (36), and inhibition by p40 of Cdc28 phosphorylation of other substrates (30). This study demonstrates that both overproduction and complete loss of p40 have dramatic consequences on the yeast cell division cycle, providing support that at least one of the in vitro functions has in vivo relevance.

The Δ*sic1* strain produces cells that frequently fail to enter the medial nuclear division step of the yeast cell cycle (35). This does not necessarily indicate that p40 has a role as a mitotic initiator, however, since arrest at this stage commonly results from activation of a G_2 checkpoint which is, in turn, a response to incomplete DNA replication or to DNA damage. The morphology of the p40 overproducer and the in vitro effects on Cdc28 protein kinase activity support, instead, a role for p40 as a negative regulator of cell cycle progression. We interpret the failure of Δ*sic1* cells to execute medial nuclear division as a consequence of premature activation of the Cdc28 kinase earlier in the cell cycle which then leads to damage that activates the G_2 arrest checkpoint. The frequent appearance of broken chromosomes in the Δ*sic1* strain may be the agent that activates the G_2 checkpoint, but we feel that it is more likely that the broken and lost chromosomes arise when cells "leak" through a prolonged G_2 arrest. Mutational loss of the *rad9* (52) and *mad2* (28) checkpoint genes also display elevated chromosomal loss rates, so this may be a common occurrence when controls coordinating cell cycle events are lost.

Although it is likely that p40 has interactions with other cellular components—the genetic results of Donovan and Johnston (11) suggest a role for *DBF2*—loss of inhibition of Cdc28 activity is the simplest explanation for the increased frequency of chromosomal aberrations in the Δ*sic1* mutant. Decreased mitotic stability is displayed by a number of *cdc28* alleles (10, 33). These alleles would be good candidates for mutants that have poor p40-Cdc28 interactions. The chromosomal damage in the Δ*sic1* strains is the type that would be expected for survivors of the mitotic catastrophe effect, such as that observed when $p34^{cdc2}$ is prematurely activated in *S. pombe* (16).

If Clb-Cdc28 complexes are the primary target of p40 action, why does p40 appear to be limited to the G_1 phase of the cell cycle (31)? Although the Clb cyclins are most closely related to the classical B-type mitotic cyclins, some *CLB* genes are expressed in S phase or even concomitantly with the *CLN* genes in late G_1 (41). Preventing premature activation of these cyclin-Cdc28 species, much as phosphorylation on tyrosine 15 prevents premature activation of MPF, could be p40's primary function. Alternatively, the appearance of p40 late in the cell cycle might indicate that p40 has a role shutting off the mitotic cyclin in preparation for cytokinesis and G_1. The genetic analyses presented in this paper could be interpreted to support either alternative, and future experiments will be needed to ascertain p40's true function. It should also be pointed out that the earlier studies on the periodicity of p40 (31) relied on the ability of p40 to be phosphorylated in anti-Cdc28 immunoprecipitations for detection. This assay would not have detected p40 had it been modified so that it could no longer be phosphorylated in vitro; therefore, the possibility that p40 is present throughout the cell cycle remains. Attempts to detect p40 in S- and M-phase cell extracts by immunoblot or immunoprecipitation have been unsuccessful (personal observations), but these methods work poorly even with G_1-phase extracts because of p40's low abundance.

These studies do not identify the process in which p40 is involved but have indicated that it is unlikely to be a component of the systems that monitor DNA replication or repair, spindle formation, mating pheromone response, nutritional response, or heat shock. A clue towards the identity of this process may come from the mother-daughter asymmetry observed in the pattern of cells that undergo permanent arrest. Several processes with a similar asymmetry have been described for yeasts, including mating type switching (49), mitotic segregation of acentromeric plasmid DNAs (32), and segregation of the spindle pole body (SPB) (50). The duplication and segregation of the SPB are attractive candidates for processes that could be monitored by a p40 checkpoint. The SPB is a complex structure that is duplicated conservatively (50) during the short time interval between START and the beginning of S phase (8), the same time interval during which the ability of p40 to be phosphorylated by Cdc28 disappears (31). The SPB is responsible for forming the mitotic spindle during mitosis, so defective SPBs would be expected to have difficulties accurately segregating chromosomes during mitosis. Because of its complexity and the short time period in which it is duplicated, SPB formation might be expected to be an error-prone process that would need to be monitored. If the SPB were assembled incorrectly, the daughter would usually receive the defective copy (50), causing subsequent inviability in an asymmetric fashion. Adding to the attractiveness, $p34^{cdc2}$ complexes have been localized to the SPB of *S. pombe* (1) and the analogous structure, the centrosomes of HeLa cells (4). Since the entire daughter cell in *S. cerevisiae* is synthesized asymmetrically, however, other processes may also be candidates for regulation by a putative p40 checkpoint.

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