An Essential Function of Yeast Cyclin-dependent Kinase Cdc28 Maintains Chromosome Stability*

Ana A. Kitazono and Stephen J. Kron‡

From the Center for Molecular Oncology and Department of Molecular Genetics and Cell Biology, the University of Chicago, Chicago, Illinois 60637

Multiple surveillance pathways maintain genomic integrity in yeast during mitosis. Although the cyclin-dependent kinase Cdc28 is a well established regulator of mitotic progression, evidence for a direct role in mitotic surveillance has been lacking. We have now implicated a conserved sequence in the Cdc28 carboxyl terminus in maintaining chromosome stability through mitosis. Six temperature-sensitive mutants were isolated via random mutagenesis of 13 carboxyl-terminal residues. These mutants identify a Cdc28 domain necessary for proper mitotic arrest in the face of kinetochore defects or microtubule inhibitors. These chromosome stability-defective cdc28\(^{\text{CST}}\) mutants inappropriately continue mitosis when the mitotic spindle is disrupted at 23 °C, display high rates of spontaneous chromosome loss at 30 °C, and suffer catastrophic aneuploidy at 35 °C. A dosage suppression screen identified Cak1, a kinase known to phosphorylate and activate Cdc28, as a specific high copy suppressor of cdc28\(^{\text{CST}}\) temperature sensitivity and chromosome instability. Suppression is independent of the kinase activity of Cak1, suggesting that Cak1 may bind to the carboxyl terminus to serve a non-catalytic role in assembly and/or stabilization of active Cdc28 complexes. Significantly, these studies implicate Cdc28 and Cak1 in an essential surveillance function required to maintain genetic stability through mitosis.

From the onset of S phase until return to G1, surveillance pathways collaborate to maintain chromosome stability, monitoring completion of DNA replication and repair and determining attachment and tension in the mitotic spindle while regulating sister chromatid cohesion and mediating chromosome condensation (1). The budding yeast Saccharomyces cerevisiae offers a powerful model system with which to dissect these multiple determinants and pathways. Several genetic screens have identified genes that are implicated in chromosome stability (2, 3) and/or kinetochore function (4–7). The yeast kinetochore is a centromere binding complex and attachment point for the plus-end of a single spindle microtubule, linking each sister chromatid to one spindle-pole body (SPB).\(^{1}\) Furthermore, the kinetochore is an assembly point for components of the spindle checkpoint, which mediates the dependence of mitotic progression on attachment and tension (8).

The cyclin-dependent kinase Cdc28 (Cdk1 and Cdc2) is the master regulator of the yeast cell cycle (9, 10). It coordinates bud emergence, SPB duplication, and DNA replication at Start and directs spindle assembly and function in mitosis. Whereas the abundance of Cdc28 is dispensable, its activity is regulated by associations with cyclins, stochiometric inhibitors, and accessory factors as well as by activating and inhibitory phosphorylations (9, 10). Even subtle changes in Cdc28 function affecting mitotic progression may impinge on chromosome stability. Indeed, mutations of several Cdc28 partners and substrates have been associated with genomic instability, whereas mutations of Cdc28 itself, such as cdc28-1N (11), cdc28-srm (12), and cdc28-5M (13), are associated with relatively nonspecific defects in chromosome segregation and/or mitotic checkpoint control. Paradoxically, current models suggest that Cdc28 and its partners are only passive effectors of the mitotic surveillance pathways (14). To dissect the role of Cdc28 in maintaining chromosome stability, we screened for cdc28 mutants that syntheitically interact with the kinetochore mutant ctf13-30 and isolated multiple temperature-sensitive alleles that no longer tolerate a kinetochore lesion. Unlike previously characterized cdc28 mutants, these alleles do not arrest at the non-permissive temperature but continue to divide, leading to mitotic catastrophe. This work identified a novel carboxyl-terminal domain of Cdc28 with an essential surveillance function that maintains genetic stability through mitosis and is critical for recognition by the Cdk1 CDK-activating kinase.

To assay responses to nocodazole (U. S. Biochemical Corp.), MAT\(^{a}\) or MAT\(^{a}\)MAT\(^{a}\) cells in the W303 genetic background (15). We obtained plasmids harboring CAK1, cak1-K31R, and cak1-D179N from R. Chum; the ctf13-30 strain from F. Spencer; the cak1::TP1 strain and cak1::SPT5316 and CKA1::SPT5316 plasmids from E. Winter; the cak1-33 ptc2::URA3 ptc3::HIS3 strain and CKA1-YEp50 and CKA1-YEp24 plasmids from M. Solomon and F. Kaldus; and the SPC42-GFP strain from M. Winey. Strain constructions and transformations were performed using standard methods (16). CKS1-3HA and CLB2-13MYC were constructed by gene replacement, introducing the 3HA or 13MYC tag from pFA6a-3HA-kanMX6 or pFA6a-13MYC-kanMX6 (17), immediately before the stop codon.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Strains, and General Yeast Methods—**All experiments were performed in MAT\(^{a}\) or MAT\(^{a}\)MAT\(^{a}\) cells in the W303 genetic background (15). We obtained plasmids harboring CAK1, cak1-K31R, and cak1-D179N from R. Chum; the ctf13-30 strain from F. Spencer; the cak1::TP1 strain and cak1::SPT5316 and CKA1::SPT5316 plasmids from E. Winter; the cak1-33 ptc2::URA3 ptc3::HIS3 strain and CKA1-YEp50 and CKA1-YEp24 plasmids from M. Solomon and F. Kaldus; and the SPC42-GFP strain from M. Winey. Strain constructions and transformations were performed using standard methods (16). CKS1-3HA and CLB2-13MYC were constructed by gene replacement, introducing the 3HA or 13MYC tag from pFA6a-3HA-kanMX6 or pFA6a-13MYC-kanMX6 (17), immediately before the stop codon.

Spot tests for viability assays were performed by placing 2.5-μl aliquots from 5-fold serial dilutions of cell suspensions onto the appropriate media, followed by incubation for 2–3 days at the indicated temperatures. Benomyl plates contained 12.5 μg/ml of the inhibitor (Sigma). UV irradiation was performed in a Stratagene cross-linker at a 100 μJ/cm² dose.

To assay responses to nocodazole (U. S. Biochemical Corp.), MAT\(^{a}\) cells were G₁-synchronized with a mating factor (Research Genetics), washed, and released into rich media at 23 °C. After 1 h, nocodazole was added to 20 μg/ml, and aliquots were taken at the indicated times. After

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1. Leukemia and Lymphoma Society Scholar. To whom correspondence should be addressed: Center for Molecular Oncology and Dept. of Molecular Genetics and Cell Biology, the University of Chicago, 924 East 57th St., Rm. 3322, Chicago, IL 60637. Tel.: 773-834-0250; Fax: 773-702-4394; E-mail: skron@midway.uchicago.edu.

‡ Leukemia and Lymphoma Society Scholar. To whom correspondence should be addressed: Center for Molecular Oncology and Dept. of Molecular Genetics and Cell Biology, the University of Chicago, 924 East 57th St., Rm. 3322, Chicago, IL 60637. Tel.: 773-834-0250; Fax: 773-702-4394; E-mail: skron@midway.uchicago.edu.
3 h, nocodazole was added to a final total concentration of 50 μg/ml, in order to extend its inhibitory activity.

To assay growth at 34 °C, exponentially growing cells at 23 °C were placed in a water bath under constant shaking, and the temperature was raised to 34 °C within 1 h. Aliquots were taken at the indicated times.

To assay the effect of CAK1 overexpression on checkpoint response, ctf13-30 strains harboring empty vector or the CAK1 plasmid were grown overnight under selective conditions. Cells were pelleted, resuspended in fresh YPD media to an \( A_{600} \) of 0.25, and incubated at 23 °C and, after 2 h (“0-time point”), the temperature was raised to 34 °C within 1 h. Aliquots were taken at the indicated times.

Cell properties were scored according to standard criteria (e.g. Ström et al., 1998). A 3 × 2 contingency \( \chi^2 \) test of independence was used to analyze the statistical significance of differences between wild-type and mutant strains in assays of cell morphology, nuclear morphology, or SPB number after different treatments. For each experiment anova was repeated at least twice with similar results. Data were not pooled, and the results of one are reported.

**DNA Content Analysis by Flow Cytometry**—Approximately 5 × 10⁶ cells were fixed in 70% ethanol overnight at 4 °C, washed once with 1 ml of water, and incubated for 2 h at 30 °C in 50 mM Tris, pH 8.0, containing 0.25 mg/ml of RNase A (Sigma). Cells were washed once with water, pelleted, resuspended in 0.2 ml of 5 mg/ml pepsin in 0.45% HCl, and incubated at 37 °C for 1 h. The cells were pelleted, resuspended in 1 ml of water, lightly sonicated, pelleted, resuspended in 1 ml of 2.5 μM Sytox Green (Molecular Probes) in 50 mM Tris, pH 7.5, incubated for 1 h at 23 °C or overnight at 4 °C, and then analyzed in a FACSCalibur flow cytometer (BD Biosciences). For each sample, data from at least 30,000 events were acquired for analysis.

**Site-directed Mutagenesis**—To create a library of strains carrying random mutations in codons 282 and 287 of CDC28 linked to a selectable marker, cells were transformed with a mutagenic PCR product synthesized by fusing a “spiked” (70% wild-type base, 10% each other base) oligonucleotide primer to the His3MX6 marker of pFA6a-2×3HA expression levels were confirmed by immunoblotting with anti-PSTAIRE antibody (Santa Cruz Biotechnology, 1:200 dilution) and analyzed by phosphorimaging (Amersham Biosciences). Parallel immunoprecipitations were run up to the washing step, and the beads were resuspended in 1 x SDS-sample buffer and boiled. Samples were loaded into 12% SDS-PAGE gels and immunoblotted with anti-PSTAIRE antibody (Santa Cruz Biotechnology, 1:200 dilution) to confirm the isolation of equal amounts of the CDC28 complexes. Cks1–3HA expression levels were confirmed by immunoblotting with anti-HA antibody (1:500 dilution). Horseradish peroxidase-linked sheep anti-mouse IgG or anti-rabbit IgG antibody (1:2500 dilution, Amersham Biosciences), Super Signal Substrate (Pierce), and Hyperfilm (Amer sham Biosciences) were used for detection.

**RESULTS**

The Carboxyl Terminus of Cdc28 Is a Determinant of Chromosome Stability—We identified a novel conserved sequence at the carboxyl terminus of Cdc28 (Fig. 1A) and implicated this domain in regulation of genetic stability and checkpoint proficiency. To study this region further, we randomly mutagenized codons 282 and 287 through 298 of Cdc28 and searched for mutations that exacerbate the phenotypes of ctf13-30, a conditionally lethal allele in a wild-type background. For each isolate, we identified a single, unseparated DNA mass and adjacent SPBs. Incubated at 32 °C, ctf13-30 cells arrest in response to detached chromosomes, accumulating in mitosis as large budded cells with a single, unseparated DNA mass and adjacent SPBs. Incubated at 32 °C, ctf13-30 grows slowly, delaying in mitosis. Both viability of ctf13-30 incubated transiently at 35 °C and growth of ctf13-30 at 32 °C depend on the spindle checkpoint pathway to prevent catastrophic mitotic progression. We mutagenized the chromosomal CDC28 allele in a ctf13-30 SPC42-GFP strain by replacing the carboxyl-terminal codons with a PCR product derived from a spiked mutagenic oligonucleotide, leaving the mutated allele adjacent to a selectable marker (19). Approximately 10% of the transformants enhanced thermosensitivity at 32 °C. By using confocal microscopy to simultaneously image cell shape and the GFP-labeled SPBs, we identified six isolates that no longer accumulate with large buds or short mitotic spindles at 32 °C. At 35 °C, ctf13-30 cells arrest in response to detached chromosomes, accumulating in mitosis as large budded cells with a single, unseparated DNA mass and adjacent SPBs. Incubated at 32 °C, ctf13-30 grows slowly, delaying in mitosis. Both viability of ctf13-30 incubated transiently at 35 °C and growth of ctf13-30 at 32 °C depend on the spindle checkpoint pathway to prevent catastrophic mitotic progression. We mutagenized the chromosomal CDC28 allele in a ctf13-30 SPC42-GFP strain by replacing the carboxyl-terminal codons with a PCR product derived from a spiked mutagenic oligonucleotide, leaving the mutated allele adjacent to a selectable marker (19). Approximately 10% of the transformants enhanced thermosensitivity at 32 °C. By using confocal microscopy to simultaneously image cell shape and the GFP-labeled SPBs, we identified six isolates that no longer accumulate with large buds or short mitotic spindles at 32 °C. At 35 °C, each of the cdc28²⁸⁻⁻° (chromosome stability) mutations confers rapid loss of viability. Cells bypass arrest yielding unbudded cells, multiply budded cells, cells with three or more SPBs, and cells that appear anucleate (Fig. 1B). To determine whether the phenotype is dependent on the kinetochore mutation, we separated the cdc28²⁸⁻⁻° mutations from ctf13-30. The single cdc28²⁸⁻⁻° mutations confer instability to an indicator mini-chromosome as evidenced by an increase in sectored and pink colonies (Fig. 1C) (5, 20).

Sequencing of the cdc28²⁸⁻⁻° alleles revealed multiple mutations, ranging from 4 to 7 of the 13 randomized residues. The mutations cluster in a sub-domain, falling particularly in residues Ala-290, Ile-291, Pro-293, Tyr-294, and Gln-296 (Fig. 1D).
The cdc28-cst3 and -cst7 mutations lead to truncations after Phe-295 and Pro-293, respectively. Only Pro-293 is mutated in all six cdc28CST mutants but to no single side-chain class. Based on the human CDK2 atomic structures, the residues altered in the cdc28CST mutants lie on the solvent-accessible surface of Cdc28 (Fig. 1E) but relatively distant from the binding sites of ATP, substrate peptide, cyclin, Cks1, or Kap1 (23–25), suggesting altered interactions with a novel regulator.

Single cdc28CST Cells Are Temperature-sensitive and Accumulate Aneuploids—Single cdc28CST mutants remain temperature-sensitive (Fig. 2A). In general, previously described cdc28 temperature-sensitive mutants arrest homogeneously at restrictive temperature and resume cell cycle progression when returned to permissive temperature. By contrast, the cdc28CST mutants do not arrest at non-permissive temperature and even short incubations decrease viability. At 25 °C, cdc28-cst2 mutant cells are slightly larger and more elongated than wild type, and at 34 °C they continue to divide but only form microcolonies (Fig. 2B). Within 3 h after a gradual shift from 23 to 34 °C, cdc28-cst2 and cdc28-cst8 accumulate misshapen, unbudded cells and with supernumerary SPBs (Fig. 2C). Between 3 and 5 h at 34 °C, flow cytometry revealed a growing fraction of apparently aneuploid cells with <1N or >2N DNA content (Fig. 2D). The increase in the <1N fraction corresponds to a similar increase in cells that apparently lack nuclei and/or chromosomal DNA by fluorescence microscopy. At 6 h, compared with wild-type cells, 8-fold more cdc28-cst2 (n = 156, p < 0.0005; Fig. 2E) and 6-fold more cdc28-cst8 (n = 214, p < 0.0005) display only background staining. Incubation at 34 °C also causes progressive loss of viability as measured by decreasing colony-forming units after plating at 23 °C (plating efficiency <1% after 10 h at 34 °C).

cdc28CST Cells Do Not Arrest upon Treatment with Microtubule Inhibitors—To further characterize the phenotype, we examined other perturbations such as treatment with the microtubule inhibitors nocodazole and benomyl. Unlike wild-type cells treated with nocodazole, which maintain a 2N DNA content through 5 h, cdc28-cst2 cells bypass arrest to perform multiple rounds of DNA replication and incomplete chromosome segregation (Fig. 3A). Likewise, rather than performing a large-budded arrest on media containing 12.5 μg/ml benomyl, cdc28CST cells continue to divide and show increased lethality. These results suggest that the cdc28CST mutants cannot maintain arrest in the presence of microtubule inhibitors and instead prematurely resume cell cycle progression to suffer mitotic catastrophe. Nonetheless, DNA damage responses are intact because UV irradiation of wild-type or mutant cells leads to similar large-budded arrest and recovery and equivalent DNA damage responses.
survival (Fig. 3B). In the same way, block and release with the replication inhibitor hydroxyurea leads to equivalent large-budded arrests followed by prompt return to S phase progression. These data are consistent with a specific defect for the cdc28CST mutants in spindle surveillance rather than in either mitotic progression or mitotic arrest. The sensitivities of the cdc28CST mutants to ctf13-30 and microtubule inhibitors are consistent with a role for Cdc28 in responding to lack of kinetochoore attachment, which normally results in spindle-assemble checkpoint arrest (1, 26–28).

High Copy Screen Identifies Cak1 as Suppressor of Temperature Sensitivity—As the cdc28CST mutations alter the predicted molecular surface but are well separated from the active site, we hypothesized that the defect might derive from impaired interactions with a protein partner. Thus, we tested whether overexpression of a known Cdc28 activator, Cks1, might partly compensate for the cdc28CST defect. Whereas overexpression of Cks1 markedly suppresses growth of the temperature-sensitive mutants cdc28-1N (Pro-250→Leu) and cdc28-4 (His-128→Tyr) at 35 °C, suppression of ctf13-30 cdc28-cst2 or ctf13-30 cdc28-cst8 is negligible (Fig. 4A) (24). To extend this paradigm, we performed a dosage suppression screen. Whereas such a strategy might reveal the partner to which cdc28CST mutants bind poorly, suppressors could include CDC28 itself, components of the relevant Cdc28 complex, upstream activators, phosphorylation targets, or parallel pathways. Thus, ctf13-30 cdc28-cst2 and ctf13-30 cdc28-cst8 strains were transformed with a high copy number genomic library and screened for enhanced thermoresistance. Isolated clones fell into several groups. As expected, wild-type CDC28 was cloned multiple times. Whereas plasmids were isolated carrying the CTF13, CBF2/NDC10, or CEP3 components of the Cbf3 kinetochore complex (29), each suppresses ctf13-30 but has no effect on cdc28CST mutants alone. Multiple clones isolated in both the cdc28-cst2 and cdc28-cst8 strains contained the CAK1 open reading frame and flanking regions of various lengths. CAK1 encodes the CDK-activating kinase that phosphorylates monomeric Cdc28 at Thr-169 and is essential for

FIG. 2. The cdc28CST mutants are temperature-sensitive and exhibit increased aneuploidy at non-permissive temperature. A, temperature sensitivity analysis. 5-Fold serial dilutions of homozygous diploid cell cultures were spotted onto rich media plates that were incubated at the indicated temperatures. B, exponentially growing homozygous diploid cells were micro-manipulated onto rich media forming a grid. Plates were incubated at 34 °C and images recorded at the indicated times. C-E, genomic instability in the cdc28CST mutants. Exponentially growing homozygous diploid cells were transferred to 34 °C and incubated for the indicated times. C, cellular morphology and SPC42-GFP SPB signal after 4 h of treatment. The arrows indicate cells with supernumerary SPBs. Bar, 5 μm. D, DNA-content profile of cells after 5 h of treatment. Note the high proportion of apparently anucleate (arrows) and/or aneuploid cells. E, proportion of cells lacking detectable nuclear DNA staining, as a measure of accumulation of anucleate cells at 34 °C. WT, wild type.

FIG. 3. The cdc28CST mutants do not arrest when treated with microtubule inhibitors. A, haploid cells were synchronized in G1, with a-mating factor, released into rich media, and allowed to re-enter the cell cycle for 1 h. Nocodazole was added to 20 μg/ml (0-h time point), and aliquots were taken at the indicated times. Cells were fixed and stained after 0–3, 3.5, 4, 4.5, 5, and 5.5 h of treatment and analyzed by flow cytometry. B, 5-fold serial dilutions of diploid cells were spotted onto rich media (untreated), rich media containing 12.5 μg/ml benomyl, or rich media and irradiated with 50 J/m² UV. Plates were then incubated at 25 °C for 3 days.
kinase activity throughout the cell cycle (30). Subcloning and retransformation revealed that CAK1 specifically suppresses cdc28-cst2 and cdc28-cst3 but not ctf13-30. High copy CAK1 has little effect on wild-type cell growth but markedly suppresses all six cdc28CST mutants at temperatures up to 34 °C (e.g. Fig. 4B). A single additional copy of CAK1 (CEN vector), like empty vector or a single copy of the mutant cak1-17, has no effect (Fig. 4C). Interestingly, high copy CAK1 does not suppress the thermosensitivity of ctf13-30 strains carrying cdc28-4 or cdc28-1N (Fig. 4D).

High Copy CAK1 Suppresses the Mitotic Surveillance Deficiency of the cdc28CST Mutants—To examine the effects of CAK1 overexpression on the cdc28CST ctf13-30 double mutants, cultures carrying empty vector or a high copy CAK1 plasmid were inoculated onto rich media and incubated at the indicated temperatures. Assays were performed in single cdc28-1N and cdc28-4 mutants (top panel) and in ctf13-30 strains carrying wild-type CDC28 or cdc28CST mutants (bottom panel). B, wild-type (WT), cdc28-cst2, cdc28-cst3, and cdc28-cst8 transformants harboring empty vector (2 μ) or the CAK1 plasmid (2 μ-CAK1) were tested for growth at the indicated temperatures. C, wild-type, cdc28-cst2, and cdc28-cst8 strains harboring low copy plasmids with no insert (CEN), a cak1-17 temperature-sensitive allele, or the CAK1 wild-type open reading frame were tested for growth at 25 and 32 °C. D, ctf13-30 strains expressing wild-type or mutant CDC28 and carrying either empty vector or the high copy number CAK1 plasmid were inoculated onto rich media and incubated at the indicated temperatures. Lack of suppression of ctf13-30 cdc28-1N or ctf13-30 cdc28-4 indicates allele specificity to the cdc28CST mutants.

Cak1 Overexpression Partially Suppresses the Catalytic Defect of the cdc28CST Mutants—If the cdc28CST mutants are poorly activated by Cak1, they may be catalytically defective. To test this, whole cell extracts from wild-type and cdc28CST mutant cells grown at 23 °C were assayed for Cdc28-associated kinase activity. Immunoprecipitates of Cks1–3HA from cdc28-cst2 and cdc28-cst8 did not contain diminished amounts of Cdc28 protein but displayed only 50 and 40% of wild-type histone H1 kinase activity, respectively (Fig. 6A). Comparable results were obtained with Clb2–13Myc-Cdc28 complexes.

Suppression by CAK1 Is Independent of Its Kinase Activity—Chun and Goebl (31) have described and characterized several cak1 point mutants. Among these, a substitution of Lys-31 for Arg (cak1-K31R) was found to display greatly reduced catalytic activity. Lys-31 is a highly conserved residue with an essential role positioning ATP in related kinases but may be dispensable in Cak1, as cak1-K31R is able to fulfill all essential functions of the kinase. A second highly conserved residue, Asp-179, participates in coordinating a magnesium ion at the active site. Substitution of this residue with asparagine (cak1-D179N) ren-
CDC28 ctf13-30 -cst2 ctf13-30 -cst8 ctf13-30

Fig. 5. Overexpression of CAK1 partially suppresses the genomic instability of cdc28-cst2 and cdc28-cst8. To assay the effect of CAK1 overexpression on checkpoint response, ctf13-30 strains expressing wild-type CDC28 or cdc28^{CST} mutants and harboring empty vector (2 μ) or the CAK1 plasmid (2 μ-CAK1) were subjected to incubation at non-permissive temperature. Cells grown overnight at 23°C under selective conditions were collected, resuspended in fresh YPD media, incubated for 2 h at 25°C (0-h time point), warmed to 34°C, and incubated at non-permissive temperature for 0.5 h. Aliquots were taken at the indicated times, and DNA content profiles were determined by flow cytometry analysis. The increase in 2N DNA content and decreased aneuploidy evident at 2–5 h in cdc28^{CST} mutants overexpressing CAK1.

\(^3\) J. Fitz Gerald, A. Kitazono, and S. Kron, unpublished results.
mutants continue to divide, become aneuploid, and lose viability, suggesting that the lethal defect is a loss of genetic stability rather than simply a defect in cell cycle progression. CAK1 was identified as a strong dosage suppressor of both the cdc28CST temperature-sensitive phenotype and chromosomal instability defects. In concert with binding of cyclin and the Cks1 subunit, CDK phosphorylation by Cak1 has a critical role in activating kinase function (30). Suppression by Cak1 may argue for a defect in cdc28CST catalytic activity per se as CAK1 overexpression in the cdc28CST mutants restored total Cdc28 histone H1 kinase activity by up to 50%. Although histone H1 kinase activity does not necessarily correlate with in vivo function, this moderate increase in catalytic activity may be sufficient to relieve the defects and confer normal thermotolerance. Yet, suppression of the cdc28CST mutants is specific, as CAK1 overexpression does not suppress the nonspecific cdc28-4 G1-arresting nor cdc28-1N G2-arresting mutants. In turn, overexpression of the CDK-activating subunit CKS1 suppresses the thermosensitivity of the cdc28-4 and cdc28-1N mutants but not the cdc28-cst2 and cdc28-cst8 mutants. Perhaps the cdc28CST defects reflect decreased Cdc28 activation by Cak1 and potentially implicate the Cdc28 carboxyl terminus in Thr-169 phosphorylation. Indeed, we found that combining the cdc28 and cak1-23 mutations led to inviability (synthetic lethality) even at 23°C, suggesting a requirement for full Cak1 activity. Nonetheless, deleting candidate Cak1 antagonists, the Thr-169 phosphatases Ptc2 and Ptc3 (35), singly or in combination does not suppress the temperature sensitivity of the cdc28CST mutants (data not shown). Strikingly, suppression by Cak1 is not dependent on its catalytic activity. Overexpression of the “kinase-dead” allele cak1-D179N restores growth in the cdc28CST mutants to levels similar to wild-type CAK1. Our favored interpretation is that the cdc28CST mutants are specifically defective in recognition by Cak1 and that the activating effect resulting from the Cdc28-Cak1 interaction does not derive exclusively from phosphorylation at Thr-169. Prolonged binding of Cak1 to Cdc28 might result in the stabilization or assembly of the activated complex, protection against the antagonistic action of phosphatases, and/or induce changes in the substrate specificity of the complex.

Why would a change in Cak1 interaction with Cdc28 specifically affect mitotic surveillance? Other than the work described here, Cak1 has yet to be implicated in a surveillance pathway or other signal transduction cascade. Nonetheless, Winter and colleagues (36) report defects in the fidelity of nuclear segregation in the cak1-17 mutant at permissive temperature. We have not confirmed other results suggesting that interaction between Cak1 and wild-type Cdc28 is biochem-

![Fig. 6. Overexpression of CAK1 partially restores catalytic activity of cdc28-cst2 and cdc28-cst8, yet suppression is independent of catalytic activity. A, anti-HA immunoprecipitates from wild-type, cdc28-cst8, and cdc28-cst2 CKS1-3HA strains harboring empty vector (2µ-Cak1 +) or the CAK1 plasmid (2µ-Cak1 −) were tested for their ability to phosphorylate the model CDK substrate histone H1 (top panel, lane 1, no anti-HA antibody control). Activity corresponds to relative 32P incorporation measured by PhosphorImager analysis, with wild type = 1.0 (lane 2). The bottom panel shows Cdc28 content by anti-PSTAIRE (Santa Cruz Biotechnology) Western blotting analysis of parallel anti-HA immunoprecipitations. B, serial dilutions of wild-type, cak1-23, cdc28-cst2, and cdc28-cst8 cells transformed with empty vector or high copy number plasmids carrying wild-type CAK1, kinase-deficient cak1-K31R, or kinase-dead cak1-D179N were tested for growth at the indicated temperatures. Note that cak1-D179N fails to complement cak1-23 but restores growth of cdc28-cst2 and cdc28-cst8, indicating that suppression is independent of Cak1 catalytic activity.]

| B | 25°C | 32°C | 35°C |
|---|------|------|------|
| 2µ | WT   | 2µ CAK1 | 2µ cak1-K31R | 2µ cak1-D179N |
| 2µ | cak1-23 | 2µ CAK1 | 2µ cak1-K31R | 2µ cak1-D179N |
| 2µ | -cst2 | 2µ CAK1 | 2µ cak1-K31R | 2µ cak1-D179N |
| 2µ | -cst8 | 2µ CAK1 | 2µ cak1-K31R | 2µ cak1-D179N |
ically stable (37), as our attempts to confirm interaction between Cak1 and either wild-type or mutant Cdc28 by co-immunoprecipitation were unsuccessful. A two-hybrid test for protein-protein interaction indicates decreased interaction between cdc28CST mutants and Cak1, but we observed comparable defects in binding to other Cdc28 partners as well (data not shown). Potentially, the cdc28CST defects are unrelated to Cak1 phosphorylation, and instead suppression is mediated only via indirect effects. Indeed, atomic models place the Cdc28 carboxyl terminus far from the Cak1 target residue Thr-169 on the activation loop (e.g. Fig. 1E), suggesting that Cak1 might need to stretch across to contact both sites on Cdc28. Our results may suggest a protein docking mechanism between Cak1 and Cdc28 like that found between an analogous pair of kinases, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase and mitogen-activated protein kinase. By studying these enzymes, Xu et al. (38) identified complementary sets of conserved residues that determine binding of Mek1 to its substrate, Erk2. Significantly, a determinant of Mek1 binding localizes to a sequence in Erk2 structurally equivalent to the domain mutated in the cdc28CST alleles. Nonetheless, we cannot rule out a non-catalytic role for Cak1 in mitotic surveillance independent from its function as a Thr-169 kinase.

Whereas a distinct checkpoint function for Cdc28 might be unanticipated, there is precedence for a single protein serving independent functions in both cell cycle progression and surveillance. In the yeast DNA polymerase ε subunit Pol2, an amino-terminal domain provides the catalytic activity, whereas the carboxyl terminus mediates a genetically separable DNA damage surveillance function (39). Further analysis of the genetic interactions of the cdc28CST mutants with known regulators of the mitotic spindle assembly and checkpoint pathways may reveal specific roles for Cdc28 and perhaps for Cak1 in kinetochore function and surveillance.

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