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Novel role for the CDK-activating kinase Cak1 in actively growing cells

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To the Graduate Council:

I am submitting herewith a thesis written by Su Hwa Kim entitled "Novel role for the CDK-activating kinase Cak1 in actively growing cells." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Ana Kitazono, Major Professor

We have read this thesis and recommend its acceptance:

Elizabeth Howell, Gladys Alexandre

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Su Hwa Kim

August 2008

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ABSTRACT

Cdc28 is the main cyclin-dependent kinase (CDK) directing the yeast cell cycle. Besides cyclin binding, Cdc28 requires phosphorylation by Cak1 (CDK-activating kinase) to achieve full activity. In previous work, our laboratory isolated carboxy-terminal *cdc28*^{CST} mutants that display high temperature sensitivity and chromosome instability. These phenotypes are suppressed by increasing the copy numbers of Cak1 in its catalytically active or inactive form. The *cdc28*^{CST} mutants are also extremely sensitive to changes in Cak1 integrity and dosage. These results indicated that besides the activating phosphorylation by Cak1, the interaction between Cak1 and the carboxyl terminus of Cdc28 is important to maintain stable and active Cdc28 complexes. In this thesis, I discuss recent results that suggest that Cdc28 is highly dependent on Cak1 levels, even in wild type cells. Because actively growing cells require cycles of high CDK activity, we hypothesize that this can be achieved only by maintaining an optimum Cdc28:Cak1 ratio. We found that alteration of this ratio by increasing Cdc28 copy numbers is deleterious, but this effect is suppressed by concomitant increase of the copy numbers of Cak1 (catalytically active or inactive). We found that there is a very good correlation between active cell growth and Cak1 levels. Based on these results we propose the following model: When cells sense depletion of nutrients and must cease dividing, Cak1 is degraded to alter the Cdc28:Cak1 ratio leading to quick inactivation of Cdc28. According to this model, Cak1 functions as an assembly factor for Cdc28 CDK complexes coordinating nutrient availability and cell growth in yeast.

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CHAPTER I - Introduction

Regulation of Cyclin-Dependent Kinases

Cyclin-dependent kinases (CDKs) are the main cell cycle regulators in all eukaryotes. Only one CDK, Cdc28, is necessary for cell cycle progression in the budding yeast *Saccharomyces cerevisiae* (Fig. 1). Activation of Cdc28 requires association with the Cks1/Suc1 subunit (16) and one of nine different cyclins, and phosphorylation of Thr169 in the T-loop (30). Negative regulation is provided by the binding of inhibitors and phosphorylation at the amino-terminal lobe by the Swe1 kinase (29). Cdc28 is expressed constitutively throughout the cell cycle but its activity is regulated by the identity of the bound cyclin subunit: Cyclins Cln1-3 promote progression through G1, and B-type cyclins (Clb 1-6) activate S-phase and mitosis. The abundance of these cyclins is regulated via transcriptional and ubiquitin-dependent proteolysis (2, 30).

CDKs are highly conserved enzymes at both the structural and functional levels. For example, human Cdk1 and Cdk2 are 60 – 65% identical to fission yeast Cdc2 and budding yeast Cdc28 (10). In 1996, Russo *et al.* revealed the mechanism of CDK activation via analysis of the crystal structure of the Cdk2-cyclin A complex (34). After binding with cyclin A, Cdk2 activity is partially increased. Cyclin A binding induces conformational changes in the Cdk2 active site that leads to exposure of Thr160 in the activation loop (Thr169 in Cdc28), allowing its phosphorylation by the CDK-activating kinase CAK. This phosphorylation induces additional 80-300 fold activation (30) by promoting more Cdk2-Cyclin A contacts that stabilize the complex and also affects substrate binding (Fig. 2) (33).

Classes of CDK-activating kinases CAK

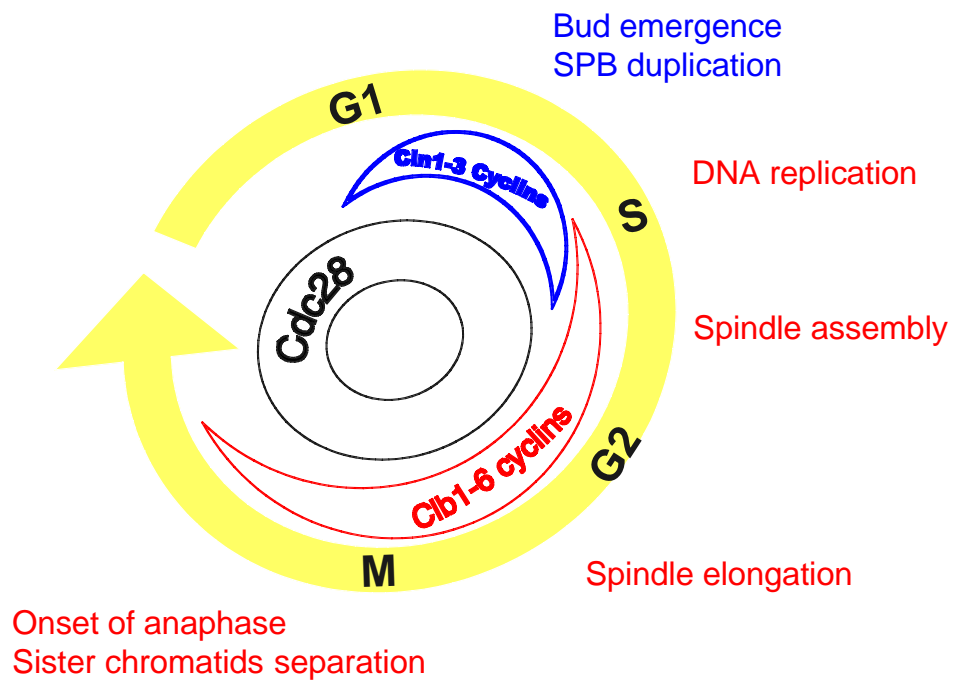


Fig. 1. Cdc28 is the main cyclin-dependent kinase (CDK) regulating the cell cycle in yeast. To promote cell-cycle progression, Cdc28 associates with a particular cyclin (Cln1-3 or Clb1-6) that confers substrate specificity and localization cues to the complex. To allow cells to exit from mitosis, Cdc28 is inactivated mainly via degradation of the mitotic cyclin Clb2.

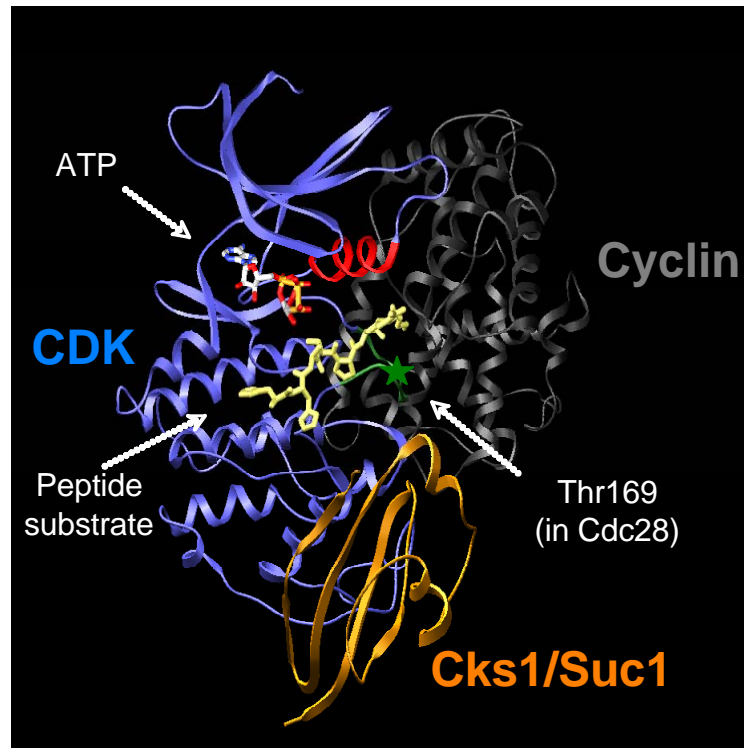


Fig. 2. Structural model of a CDK-cyclin complex. The CDK subunit is shown in blue, the cyclin subunit in gray, and the Cks1/Suc1 activator in dark yellow. The residue phosphorylated by the CAK enzymes is indicated by the green star (Thr169 in Cdc28), and the activation or T-loop is shown in green. The binding sites of ATP and the peptide substrate are also indicated. The PSTAIRE helix (red) is present in most CDKs and functions in cyclin binding (34).

There are two classes of CAKs: monomeric Cak1 from budding yeast and the trimeric complex (Cdk7/cyclin H/MAT1) from vertebrates. CDK activation via phosphorylation by CAKs has two alternative pathways: Cdk7/cyclin H/MAT1 phosphorylates CDKs that are already associated with cyclins, and Cak1 phosphorylates monomeric Cdc28. These two CAKs also have different localizations and roles. Cdk7/cyclin H/MAT1 localizes to the nucleus but Cak1 localizes mainly in the cytoplasm (22). The trimeric CAK is also involved in transcription as part of the transcription factor II-H complex, which phosphorylates the C-terminal domain (CTD) of the large subunit of RNA polymerase II. In *S. cerevisiae*, Kin28 is the most similar to Cdk7 with 47% identity and the binding partner of Kin28 is Ccl1, which is related to cyclin H. Like the trimeric CAK, Kin28 and Ccl1 are subunits of the transcription factor II-H complex and Kin28 is the CTD kinase, but it does not possess CAK activity (6).

Besides *S. cerevisiae*'s Cak1, other monomeric CAK activities have been reported in *S. pombe* (Csk1), *C. albicans* (Cak1), and *A. thaliana* (Cak1At). Interestingly, these monomeric enzymes share several properties: All these monomeric CAKs phosphorylate cyclin-free CDKs, as does Cak1 in budding yeast (40).

Cak1 is an unusual kinase and has various functions

Most protein kinases have a glycine loop characterized by the motif GXGXXG (where X is any amino acid), located near the amino terminus of the kinase domain (Fig. 3). The glycine residues provide flexibility and help the loop to fold over the nucleotide, thereby stabilizing the ATP molecule. In 1996, Solomon and colleagues compared the amino acid sequences of Cak1 with other kinases, finding that Cak1 lacks a glycine loop.

```

          GXGXXG          K
hsCdk7 : MALDVKSRAKRYERLDFLGGQFATVMKARDKNINQ..IVAIKKKKGHRSEARDGINRTALREIKLLELS..HP : 72
scKin28 : .....MKVMMEYTRKKVGGSTYAVVVLGCOHSTGR..KIINERKT...SEFRDGLDMSAIREVKYLQEMC..HP : 64
spCsk1 : .MKSVGHPVPLTDHRIHDTISSEVVGGERKNRK..LYVINVGGVFKRPPHD.....AMFGKLILESIG..HP : 66
scCak1 : .....MRDSDIITHCQLVKSTRTARIYRSDTYALICHADEIDIPPH.....AKFEVSLINRLGNKCK : 59

hsCdk7 : NITGLLEAFGHKSN..ISLVEDEMETDIEVLIKD.....NSLVLTG.....SHIK : 115
scKin28 : NVIELIIEIMAYDI..ENLVLEELPTDIEVVIKD.....KSLIFTE.....ADIK : 107
spCsk1 : HIERIVLSEIDNEAGSVLITSEKSPVLSDUMDE.....ISIDTK.....C : 107
scCak1 : HILPFLLESKATDNND..LILLFPREEMHIYEPNOMHYKRRRRKNPYDILLNPSIFIVADEPVCKYTNCLDVNRYSL : 134

          D
hsCdk7 : AYMMITTCGLELYIHOHILHRDLKPNLLLD....ENGVTRIADFGI...AKSFGS-NR...AYTHGVVTRMYR : 179
scKin28 : AYMMITTCGVYHCHRNELHRDLKPNLLIFS....PDGCIKVADFGI...ARAIPAGHG...ILTSNVVTRMYR : 171
spCsk1 : KIVLQISSALENTEKHGILHRDIHPNLLIDS....MNEPAYLSDFSI...ANSKQHGGEVQELIPQIGTGHYR : 175
scCak1 : SEFROMVEGIAFLHENKIIHRDIKEQNDMTNNTSTVSPKTYIIDFGISYDMANNSQTSAPMDSKVTDISTGIYK : 210

          D
hsCdk7 : ABELLFGARMYGVGVDMMAVGCILAELLR.....VEFLPGI.....SDLDQLTRIFETLGTPREEQWED : 239
scKin28 : ABELLFGARHYTSAIDTMSVGVIFAEIMLR.....IEVLEGG.....NOVDQEVTERALGTPIDRDWFE : 231
spCsk1 : AIELTLFGCHSYSGHEVLRDTFGILLAEIFS.....NQALFDDGSSEGWP...SEIRLTSSIIQLGTENFPMWFE : 241
scCak1 : ABEVLFGVRCYDGEVDVWSLLIISQNFQRETSMGHVEAMIDDGSDDMNSDGSIFRLICSTFPERLGIPISCKWFE : 286

hsCdk7 : MCSLPDVTTEKSFPG...IPLHHIFSAAGDDLLDLIOGIFLPNFCARITATCAKMKMFSNRPGSTPGCQLPRPNC : 312
scKin28 : VSSFMTYAKLQIYPPPSRDELAKRFIAASEYALDFMCGILTNPKRRWTAVCLESDFKELPPS..... : 296
spCsk1 : LSLFPDVKKFIPEYF...PKPWSEILPSVDTSIQYIVSHLV.....YENRASE..... : 288
scCak1 : VACHGSVLAEVGMFGADGDGKYVLDQEKDVQISIVERNPRUEIADVKVKCK.....EINCILG..... : 346

hsCdk7 : PVETLKECSNBAIAIKRKRTEALEQGGLPKKLIF : 346
scKin28 : .....SDPSIKIRN..... : 306
spCsk1 : ...SFVIESFEKVSARLSQYA..... : 306
scCak1 : .....MVSEFS..PNERMNCQRILQELEKP.... : 368

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Fig. 3. Sequence comparison of Cak1 with Cdk7 (human [*hs*]), Kin28 (*S. cerevisiae* [*sc*]), and Csk1 (*S. pombe* [*sp*]). The GXGXXG motif is indicated in red. The invariant lysine residue is indicated in green. Residue Asp179 (shown in orange) is critical for magnesium binding and its mutation abolishes catalytic activity (*cak1*-KD). Mutation of residue Asp226 (in blue) to Ala causes temperature sensitivity in yeast (*cak1*-23). The residues shown in a black background are present in all four kinases, and the ones in gray are present in three or two of the sequences.

Cak1 also contains non-conservative replacements at 4 of 15 highly conserved amino acid positions in protein kinases. Interestingly, Cak1 is not affected by either mutation or deletion of this region (11). Another highly conserved feature of protein kinases is the invariant lysine residue, located carboxy-terminal to the glycine loop within the ATP-binding pocket (Lys31 in Cak1). This residue is required for activity, and interacts with the α - and β - phosphates of ATP to ensure proper alignment of the triphosphate chain. In contrast, Cak1 functions even with a truncation of its first 31 amino acids, including the glycine loop and Lys31(5, 17). Therefore, Cak1's nucleotide-binding pocket differs significantly from that of most other kinases and its identification remains pending.

Cak1 has various functions. Besides phosphorylating Cdc28, Cak1 is involved in spore wall morphogenesis (41), and phosphorylation of the Kin28, Bur1, and Ctk1 CDKs that regulate transcription as CTD-kinases. Cak1 also interacts functionally with Paf1 and Ctr9, the two components of the PAF1 elongation complex required for histone modifications, and with the protein phosphatase Ssu72 via Ctk1 and Bur1 (14). However, Cak1 is only essential for phosphorylating Cdc28 (39), as demonstrated by the isolation of *cdc28* mutants harboring several substitutions including Thr169Glu that remain viable in the absence of Cak1 (7, 8).

Mutagenesis of the carboxyl terminus of Cdc28

In wild type cells, treatment with microtubule toxins such as benomyl or nocodazole, or presence of non-functional kinetochores leads to activation of the spindle assembly checkpoint (1).

Kinetochores are large protein complexes that assemble at centromeric regions and serve as the site of attachment for the spindle microtubules. Accordingly, their correct function is critical to facilitate separation and segregation of the duplicated chromosomes (43). The role of the spindle assembly

checkpoint is to prevent spontaneous chromosome loss by coordinating the assembly and disassembly of the mitotic apparatus, and the timing of separation of the duplicated chromosomes (27). Our laboratory has previously established a role for CDKs in spindle assembly checkpoint regulation via the isolation of *cdc28* mutants that are defective in the checkpoint response (24, 25). These mutants show chromosome instability and enhanced benomyl sensitivity similar to checkpoint mutants. Residues Arg287 and Arg288 in the carboxyl terminus of Cdc28 were substituted in some of the mutants, which led our attention to the region. Introduction of more substitutions at the carboxyl terminus of Cdc28 led to stronger phenotypes (*cdc28*-CIN1: Arg287Ser, His292Glu, Pro293Leu, Tyr294Ile, Phe295Stop) (24). These data suggest a critical role for the carboxyl terminus of Cdc28 that may contribute to the checkpoint response. Furthermore, while sequence alignments of several CDKs show that their carboxyl termini are highly conserved (Fig. 4) (25), a specific function has not been reported for this region.

To further investigate the role of the carboxyl terminus of Cdc28 in maintaining chromosome stability, we searched for mutations in Cdc28 that enhance the temperature sensitivity of the kinetochore mutant *ctf13-30* (32) (see Table 1 for a description of all mutants used in these studies). The reasoning behind this screen was that cells harboring defective kinetochores rely for viability on pathways that prevent progression through mitosis (such as the spindle assembly checkpoint). If a component of one such pathway were non-functional, the *ctf13-30* mutant cells would progress through mitosis with lethal consequences.

<i>scCdc28</i>	DPIN	RISARRAAIHYPFQE	S-----	-----	298
<i>hsCdk1</i>	DPAK	RISGKMALNHPYFND	LDSQIKKM-----	-----	297
<i>spCdc2</i>	DPAH	RISAKRALQQNYLRD	FH-----	-----	297
<i>hsCdk2</i>	DPNK	RISAKAALAHPPFFQD	VTKPVPHLRL-----	-----	298
<i>hsCdk4</i>	NPHK	RISAFRALQHSYLHK	DEGNPE-----	-----	303
<i>hsCdk6</i>	NPAK	RISAYSALSHYPFQD	LERCKENLDSHLPPS	QNTSELNTA	326

Fig. 4. The carboxyl termini of CDKs are highly conserved. Cdc28 is from *S. cerevisiae*, Cdk1, Cdk2, Cdk4 and Cdk6 are human CDKs, and Cdc2 is from *S. pombe* (25).

Table 1. Mutants used in this study

Name	Definition	Reference
<i>cdc28-cst8</i>	High chromosome instability, sensitive to high temperatures and microtubule inhibitors (Ala290Gly His292Leu Pro293Ala Gln296Ser)	(25)
<i>cdc28-cst2</i>	High chromosome instability, sensitive to high temperatures and microtubule inhibitors (Ala290Gly Ile292Lys Pro293Ser Tyr294Ser Ser298Pro)	(25)
<i>mad1</i>	Spindle checkpoint component, high sensitivity to microtubule inhibitors (<i>mad1-181</i> allele, mutation unknown)	(18, 38)
<i>mad2</i>	Spindle checkpoint component, high sensitivity to microtubule inhibitors (deletion)	(24)
<i>cdc28-T169A</i>	Inactive, lethal, unphosphorylatable by Cak1 (Thr169Ala)	(23)
<i>ctf13-30</i>	Kinetochore component. Temperature sensitive and high chromosome loss (Lys146Glu)	(9, 32)
<i>cak1-KD</i>	Catalytically inactive, mutation in magnesium binding site (Asp179Asn)	(5)
<i>cak1-23</i>	Cak1 temperature sensitive mutant allele (Asp226Asn)	(12)

For the screen, codons 282, and 287 through 298 in *CDC28* were randomly mutated via PCR (shown in boldface in Fig. 4). Six mutants were isolated that bypass the mitotic arrest yielding unbudded cells, multiply-budded cells, and cells that appear anucleate (25). These mutants show high rates of chromosome loss therefore, we called this mutant *cdc28*^{CST} (defective in maintenance of chromosome stability). Sequence analysis showed multiple mutations, ranging from 4 to 7 of the 13 carboxy-terminal residues, particularly in residues Ile291 and Pro293 (Fig. 5) (25). Based on the human Cdk2 structure, the mutated residues in the *cdc28*^{CST} mutants localize to the solvent-accessible surface but distant from the binding sites of ATP, substrate peptide, cyclin and Cks1 (4, 33, 37).

***cdc28*^{CST} mutants exhibit microtubule inhibitor sensitivity**

The *cdc28*^{CST} mutants show temperature sensitivity forming only microcolonies at high temperatures, and at 25°C, the cells are slightly larger than those of wild type. On the other hand, when treated with benomyl, wild type cells arrest in mitosis and as they recover and/or the effect of the drug diminishes, most cells are able to divide slowly and form colonies. Spindle assembly-checkpoint mutants fail to arrest and enter anaphase with incomplete spindles, causing chromosome missegregation and cell death (26). As expected, the *cdc28*^{CST} mutants are sensitive to benomyl to levels comparable to those of the checkpoint mutants *mad1* and *mad2* (27) (Fig. 6).

High copy numbers of Cak1 suppress the temperature sensitivity of the *cdc28*^{CST} mutants independently of Cak1's catalytic activity.

We hypothesized that the temperature sensitivity of the *cdc28*^{CST} mutants might derive from impaired interaction with a regulator. To attempt identification of this regulator(s), we performed

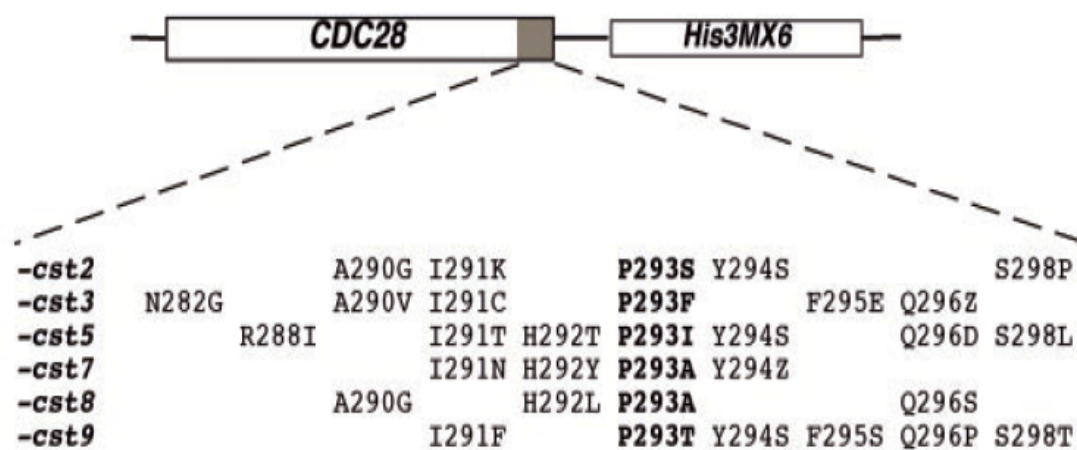


Fig. 5. Carboxy-terminal *cdc28*^{CST} mutants harbor multiple substitutions. The diagram shows the *CDC28* chromosomal locus with the residues altered in the isolated mutants. Also shown is the insertion site of the marker *His3MX6*, which allowed selection of the transformants (25).

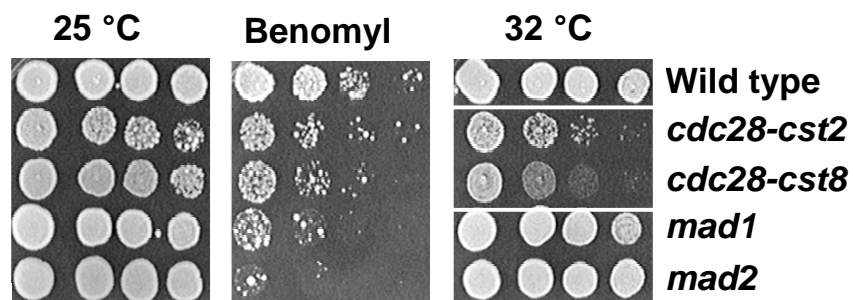


Fig. 6. Carboxy-terminal *cdc28* mutants are sensitive to the microtubule inhibitor benomyl and high temperatures. Wild type and mutant strains were serially diluted and aliquots from each dilution were spotted on three different plates containing rich media only, or supplemented with the microtubule inhibitor benomyl (12.5 µg/ml). Mad1 and Mad2 are two main components of the spindle assembly checkpoint and in their absence, cells become “mitotic-arrest deficient” and sensitive to benomyl. The plates were incubated for 2 – 3 days at the temperatures indicated in the figure. The plate containing benomyl was incubated at 25 °C (25).

a dosage suppression screen based on the temperature sensitivity of the mutants. *cdc28-cst2* and *cdc28-cst3* strains were transformed with a high copy plasmid genomic library and analyzed for enhanced thermoresistance. Several plasmids harboring *CAK1* were found to suppress the temperature sensitivity of both *cdc28*^{CST} mutants.

Chun and Goebel described and characterized several *cak1* point mutants. One of the mutations in the residue responsible for coordinating a magnesium ion at the active site, Asp179Asn (Fig. 3), caused Cak1 to be catalytically inactive (Cak1-KD) (5). We used this mutation to test whether Cak1's catalytic activity is essential for the suppression of the *cdc28*^{cst} mutants. Besides the wild type and the *cdc28*^{CST} strains, we also used a temperature sensitive *cak1-23* mutant as a control (Asp226Ala, Fig. 3). The strains were transformed with high copy plasmids harboring *CAK1* or *cak1-KD*. Interestingly, we found that overexpression of *cak1-KD* suppressed all of the *cdc28*^{CST} mutants and as expected, did not complement *cak1-23* (Fig. 7) (25). Based on these preliminary data, we concluded that Cak1's role regulating Cdc28 function not only involves phosphorylation of Thr169, but also binding to its carboxyl terminus (Fig. 8).

In this thesis, I describe recent results that suggest that maintaining an optimum Cdc28:Cak1 ratio is crucial in actively growing cells. Alteration of this ratio by increasing the copy number of the *CDC28* gene by one or two causes slow growth in wild type cells. This defect is suppressed by concomitant increase of the copy numbers of Cak1 (catalytically active or inactive), indicating that Cdc28 depends on Cak1 binding to remain active and stable. These data suggest that one mechanism by which yeast cells regulate their division rates is by allowing presence of high Cak1 levels only when high CDK activity is required. Supporting this model, we have also found that only actively

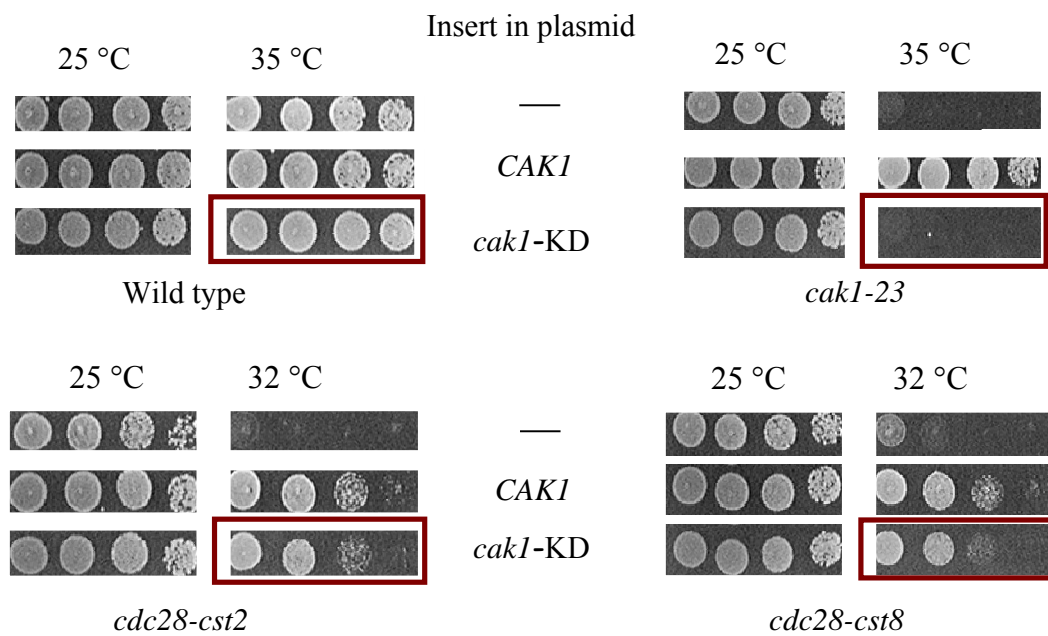


Fig. 7. High copy numbers of catalytically active or inactive *CAK1* suppress the temperature sensitivity of the *cdc28*^{CST} mutants. The panels show wild type, and temperature sensitive *cak1* and *cdc28*^{CST} mutants transformed with empty high copy plasmid control (“—”), and plasmids harboring wild type *CAK1* or the catalytically inactive allele *cak1*-KD (kinase dead). Cultures of similar densities were serially diluted, aliquots spotted, and plates incubated at the indicated temperatures (25).

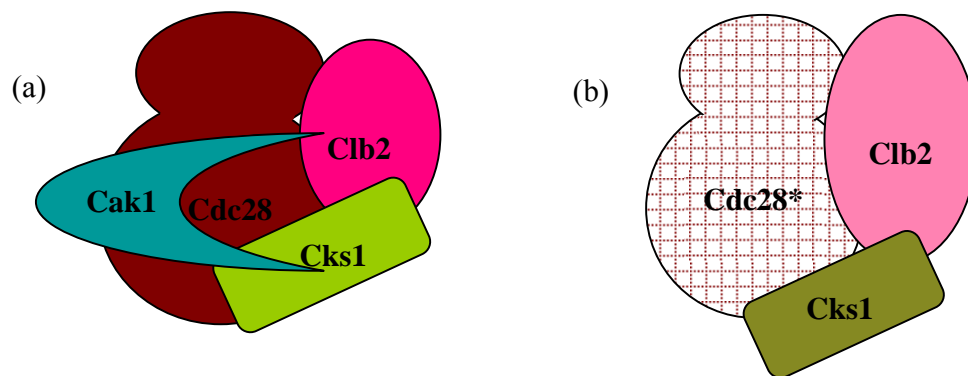


Fig. 8. Preliminary model. (a) Cak1 binds to the carboxyl terminus of Cdc28 functioning as an assembly factor that stabilizes the CDK complexes to ensure presence of high kinase activity. (b) Inability of Cak1 to bind to Cdc28 because of mutations in Cdc28 results in unstable complexes and overall low CDK activity.

growing cells display high Cak1 protein levels, and that these levels dramatically decrease as nutrients are depleted and cells cease dividing.

CHAPTER II-Materials and methods

Plasmids and yeast strains

All procedures use cells of the W303 genetic background. Plasmids harboring *cdc28*-T169A and the *cak1-23* strain were gifts from M. Solomon (Yale University) (23). *pGAL1>GST-CAK1*, *CAK1* and *cak1*-KD plasmids were provided by K. Chun and M. Goebel (Indiana University) (5). We use the “>” symbol to indicate the promoter that controls expression of a gene or fusion.

Analysis of the effect of altering copy numbers was performed using high or low copy plasmid vectors. High copy plasmids are derived from the endogenous 2 μ plasmid, which can accumulate up to ~60 – 100 copies per cell. Low copy plasmids harbor centromeres (*CEN*) that allow tight segregation during cell division and are usually present in 1 – 2 copies per cell (36).

Plasmids *pGAL1>CDC28*, *pGALS>CDC28*, and *pGAL1>CAK1* (without epitope tags) were constructed by amplifying the respective sequences using pFA6a-kanMX6-*pGAL1* and genomic DNA as templates. The flanking restriction sites were designed as *SalI* and *BamHI*. To make the *pGAL1>CDC28* and *pGALS>CDC28* constructs, the *GAL1* and *GALS* promoter regions were amplified using the SAL1GAL1FW and SAL1GALSFW primers respectively, and the CDC28RV primer. The *CDC28* open reading frame (ORF) was amplified using primers CDC28FW and CDC28BamHI. These two polymerase chain reaction (PCR) fragments were then fused together via their overlapping 25 bp ends using the outer most forward and reverse primers. The final fusion-PCR products were cloned into pRS315 (*CEN LEU2*, *CDC28* plasmids).

A similar strategy was used to construct the *pGAL1>CAK1* and *pGALS>CAK1* plasmids. The *GAL1* or *GALS* promoter region was amplified using the SAL1GAL1FW or SAL1GALS, and the CAK1RV primers. The *CAK1* ORF was amplified using CAK1FW and CAK1BamHI primers. The final fusion-PCR products were cloned into pRS316 (*CEN URA3*, *CAK1* plasmids).

Determination of transformation efficiencies

Wild type, *cdc28-cst2*, and *cdc28-cst8* cells were first transformed with pRS202 (2 μ *URA3*) plasmids harboring no insert, *CAK1*, or *cak1*-KD. Single colonies were obtained on glucose plates lacking uracil, grown in selective media, and normalized by measuring OD_{600nm}. Using suspensions of identical cell densities, a second transformation was performed with *CEN TRP1* plasmids harboring *CDC28*-HA or *cdc28*-T169A-HA, using the same amounts of DNA. After the final transformation, cells were briefly sonicated to disrupt aggregates. Numbers of cells were determined by manual counting using a hemacytometer. After normalization of cell densities, suspensions were serially diluted 5-fold with water and spotted onto media lacking uracil or both uracil and tryptophan. Plates were incubated for 2 to 4 days at 25 °C.

Effects of overexpressing *CDC28* and *cak1*-KD in wild type and the *cak1-23* mutant

Wild type and *cak1-23* mutant strains (12) were co-transformed with a low copy *CEN LEU2* plasmid harboring no insert ("—") or *GALS>CDC28*, and a high copy *URA3* plasmid harboring no insert ("—") or *cak1*-KD. After transformations, single colonies from each of the combinations were grown on glucose plates lacking both leucine and uracil to select for presence of both plasmids. These cultures were then grown in similar selective media containing 2% raffinose for 3 - 4 days at 25 °C until saturation, to ensure similar cell densities. Cultures in raffinose allowed derepression of the *GAL* promoter. Saturated cultures were serially diluted (3 fold), and spotted onto plates lacking uracil and leucine and containing either glucose (*GAL* promoters OFF), or 2% galactose and 2% raffinose (*GAL* promoters ON). The plates were incubated at 25 and 30 °C for 2-3 days.

cak1-KD was overexpressed in *cak1-23* and wild type cells. Cells were co-transformed with high copy *URA3* plasmid harboring empty vector and low copy *CEN LEU2* plasmid harboring *GAL1>cak1*-KD using the same strategy as above. The plates were incubated at 25 and 32 °C for 2 - 3 days.

Protein extract preparations and Western blotting

To examine Cak1 protein levels as cells grow and nutrients become limited, wild type cells expressing a GST-Cak1 fusion under the control of the *GAL1* promoter were grown in media containing 2% raffinose. Cells were diluted, treated with 2% galactose and aliquots collected in a time course for 34 hours. Cells were centrifuged and the pellets washed twice with 1X PBS 10% glycerol (PBSgly), and kept at -80 °C until use. Cells were disrupted in PBSgly containing 1mM phenylmethane sulfonylfluoride and 1x protease inhibitor cocktail (Roche) by vortexing with glass beads for 10 min at 4 °C. Suspensions were then centrifuged for 30 min at 4 °C to obtain the clear supernatant. Protein concentrations were determined using the Bradford method (ADV01 reagent from Cytoskeleton Inc). Aliquots containing 100 µg proteins were separated in 12% SDS-PAGE gels.

Two strategies were implemented to avoid non-specific proteolysis of Cak1 during sample processing: Use of a strain lacking vacuolar proteases due to deletion of the *pep4* gene, and preparation of the protein extracts under denaturing conditions (13). *pep4::kanMX6* cells were transformed with *pGAL>GST-CAK1*, or empty pEG(KG)His vector expressing only GST as control. Cells were grown in 2% raffinose containing media, diluted, and treated in 2% galactose. Aliquots were collected at 3 hour intervals for 24 hours. Frozen pellets were resuspended in 20% trichloroacetic acid and vortexed with glass beads for 10 min. Pellets were collected by centrifugation, washed with 90% acetone three times, mixed with sample buffer, briefly sonicated to

facilitate suspension, and boiled for 5 min. Aliquots containing 50 or 10 µg of proteins (GST-Cak1 or GST expressing cells, respectively) were used for Western blotting.

In all cases after SDS-PAGE, the separated proteins were transferred to nitrocellulose membranes. Equal loading was confirmed by staining the membranes using the MemCode kit from Pierce and/or using an anti-Adh1 antibody (1:20000, Calbiochem). The MemCode kit allows reversible staining proteins on membranes and according to the manufacturer, has a sensitivity of up to 25 ng. Cak1 was detected using polyclonal anti-Cak1 antibodies (1:4000, provided by P. Kaldis, NCI), or a monoclonal anti-GST antibody (1:5000, Novagen) when expressed as a GST fusion. Cdc28 was detected using a polyclonal anti-PSTAIRE antibody (1:2500, Santa Cruz), which also recognizes the Pho85 CDK (top band in shown blots). Horseradish peroxidase -conjugated goat anti-mouse IgG or anti-rabbit IgG antibody and Super-Signal Substrate (Pierce) were used for detection.

CHAPTER III-Results

The *cdc28*^{CST} mutants are highly sensitive to changes in Cak1 integrity and dosage.

In previous studies, we found that increasing dosages of *CAK1* suppress both the temperature sensitivity and chromosome instability of the *cdc28*^{CST} mutants (25). Since Cak1 and Cdc28 proteins interact, a corollary of this hypothesis is that disrupting Cak1 function would have a deleterious effect in the *cdc28*^{CST} mutants. First, we tested for genetic interactions between the *cdc28*^{CST} and *cak1* mutations (*cak1-4*, *cak1-17* and *cak1-23*). Haploid cells harboring both mutations could not be obtained, suggesting that a combination of *cdc28*^{CST} and *cak1* mutations results in inviable cells (synthetic lethality). Furthermore, similar synthetic effects were observed when the chromosomal copy of *CAK1* was modified to express carboxy-terminal fusions with 13-Myc or 3-HA. Presence of these epitope tags in an otherwise wild type cell caused only mild growth defects.

Interestingly, the *cdc28*^{CST} mutants also lose viability when transformed with plasmids harboring *cdc28*-T169A, an unphosphorylatable (thus inactive) allele in which Cak1's target residue Thr169 has been substituted for Ala. For these studies, wild type and the *cdc28*^{CST} mutant strains were transformed with plasmids harboring no insert, wild type *CDC28* or *cdc28*-T169A. These are centromeric plasmids (1-2 copies per cell) and carry a *TRP1* marker for selection. There was no difference between wild type and *cdc28*^{CST} mutants when transformed with empty vector or wild type *CDC28* plasmids; however, transformation efficiencies of the *cdc28*^{CST} mutants significantly decreased when transformed with *cdc28*-T169A (Fig. 9). These results suggest that unphosphorylatable *cdc28*-T169A binds to and sequesters Cak1, thereby lowering its overall dosage and causing a dominant negative effect only in the *cdc28*^{CST} mutants. To examine if the deleterious effect in the *cdc28*^{CST} mutants expressing *cdc28*-T169A is due to titration of Cak1, the assay was repeated in the presence of high copy numbers of *CAK1* (Fig. 10).

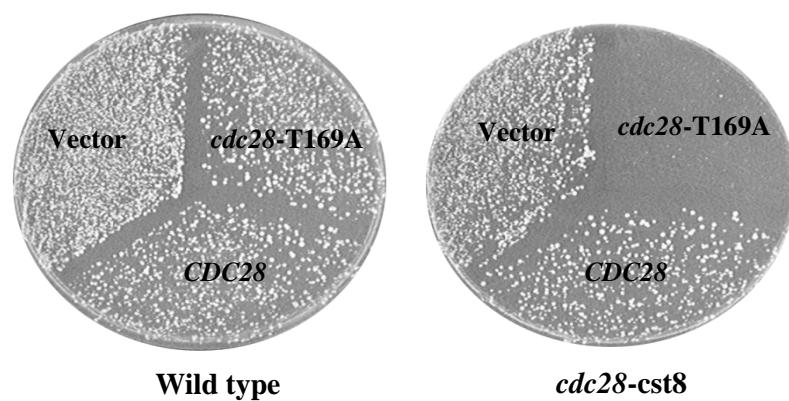


Fig. 9. Only *cdc28*^{CST} mutants harboring the *cdc28-T169A* plasmid show reduced transformation efficiency. Wild type and *cdc28*^{CST} mutants were transformed under identical conditions, with either empty vector or the same vector harboring wild-type *CDC28* or the unphosphorylatable allele *cdc28-T169A*. The transformation mixes were spread on medium lacking tryptophan and the plates were incubated at 25 °C for 3-4 days.

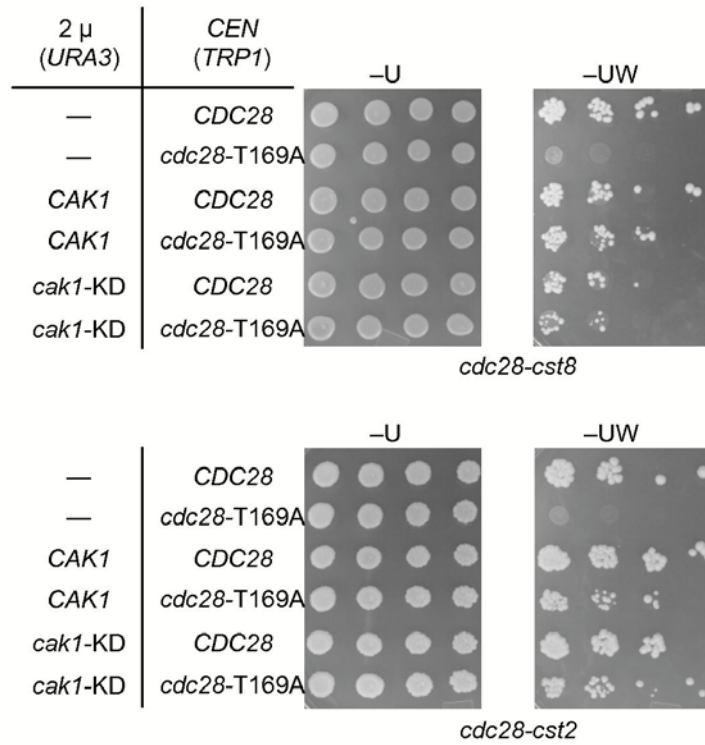


Fig. 10. High-copy numbers of *CAK1* or *cak1-KD* suppress the deleterious effects of a plasmid harboring *cdc28-T169A* in the *cdc28*^{CST} mutants. The mutants were first transformed with high copy *URA3* plasmids carrying no insert “—”, wild type *CAK1*, or *cak1-KD*. Single colonies of these transformants were then grown in media lacking uracil, and subjected to a second transformation with single copy *TRP1* plasmids carrying either wild type *CDC28* or the *cdc28-T169A* allele. The cell densities in the transformation mixes were normalized and the obtained suspensions serially diluted. Aliquots from each of the dilutions were then spotted on media lacking uracil or both uracil and tryptophan. Pictures were taken after 2- 3 days incubation at 25 °C.

First, the *cdc28*^{CST} mutants were transformed with high copy *URA3* plasmids carrying no insert, wild type *CAK1*, or catalytically inactive *cak1*-KD. Selected transformants were then normalized and subjected to a second transformation with the same amount of *CEN TRP1* plasmid carrying either wild type *CDC28* or *cdc28*-T169A. The final transformation mixes were normalized for cell densities and serially diluted 5-fold. Cells were spotted on media lacking uracil ("-U") or both uracil and tryptophan ("-UW"). Plates were incubated at 25 °C for 2 - 3 days. Similar growth on the -U plates confirmed that equal numbers of cells were spotted. On the -UW plates, we examined the transformation efficiencies for the *CDC28* and *cdc28*-T169A plasmids. High copy numbers of either *CAK1* or *cak1*-KD increased the transformation efficiencies in the *cdc28*^{CST} strain ~ >10-fold. These results suggest that Cak1 titration causes the deleterious effects due to expression of unphosphorylatable Cdc28 in the *cdc28*^{CST} mutants.

Maintenance of Cdc28:Cak1 ratio is crucial for normal growth in wild type cells

In the course of these studies we noticed that wild type cells consistently exhibited low transformation efficiencies with single-copy plasmids harboring either *CDC28* or *cdc28*-T169A. The number of colonies for these transformations was 3- to 5- fold lower than in the empty vector controls. We found that the lower transformation efficiencies were suppressed by high copy numbers of *CAK1* or *cak1*-KD (Fig. 11). This result suggests that maintenance of an optimum Cdc28:Cak1 ratio is required to support active cell growth. Cdc28 complexes are stable and exhibit highest CDK activity only in the presence of optimum Cak1 levels. Accordingly, alteration of this ratio by increasing copy numbers of Cdc28 results in formation of unstable complexes because Cak1 levels become limited. In these conditions, a competition then ensues between stable and unstable Cdc28

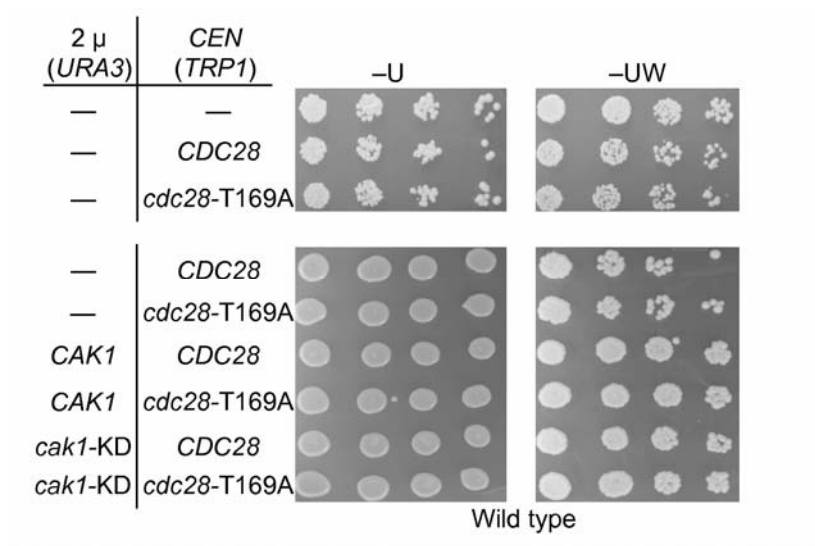


Fig. 11. Wild type cells show a deleterious growth effect with a *CEN* plasmid carrying *CDC28* or *cdc28-T169A*, but this effect is suppressed by increasing *CAK1* or *cak1-KD* copy numbers.

Wild type cells were sequentially transformed as indicated in Fig. 10

for activators such as cyclins and/or Cks1, causing an overall lowering of CDK activity and thus affecting growth rates and viability.

To further investigate the effect of altering the Cdc28:Cak1 ratio, we implemented a strategy that combines use of inducible promoters and a *cak1* mutant that exhibits low Cak1 protein levels: To overexpress Cdc28 and Cak1, we used the inducible *GALI* promoter and its weaker derivative *GALS* (20). To estimate the protein levels achieved when using these promoters, we transformed wild type cells with plasmids that allow overexpression of Cdc28 under the control of the *GALI* or *GALS* promoter (*pGALI>CDC28* or *pGALS>CDC28*). The respective transformants were grown overnight in raffinose and treated with galactose for 6 hours. Protein extracts were prepared and analyzed by western blotting using an anti-PSTAIRE antibody (Fig. 12). Under these conditions, we estimated that the Cdc28 levels were at least two times higher when using the *GALI* promoter (quantified by Image J soft program).

For these studies, we also included the *cak1-23* mutant, which exhibits significantly lower Cak1 protein levels than the wild type strain at both permissive and restrictive temperatures (Fig. 13). Wild type and *cak1-23* strains were grown overnight at room temperature and then shifted to 34 °C. Aliquots were taken at the indicated times, and protein extracts prepared and analyzed by western blotting using a monoclonal anti-Cak1 antibody. To confirm equal loading, the membrane was stripped and treated with an anti-Adh1 antibody (Adh1 is constitutively expressed and is one of five alcohol dehydrogenases in yeast (28)).

To examine the effect of overexpressing Cdc28 in a regulated manner, we compare growth rates of wild type and *cak1-23* expressing varying levels of Cdc28 (Fig. 14). We transformed the strains

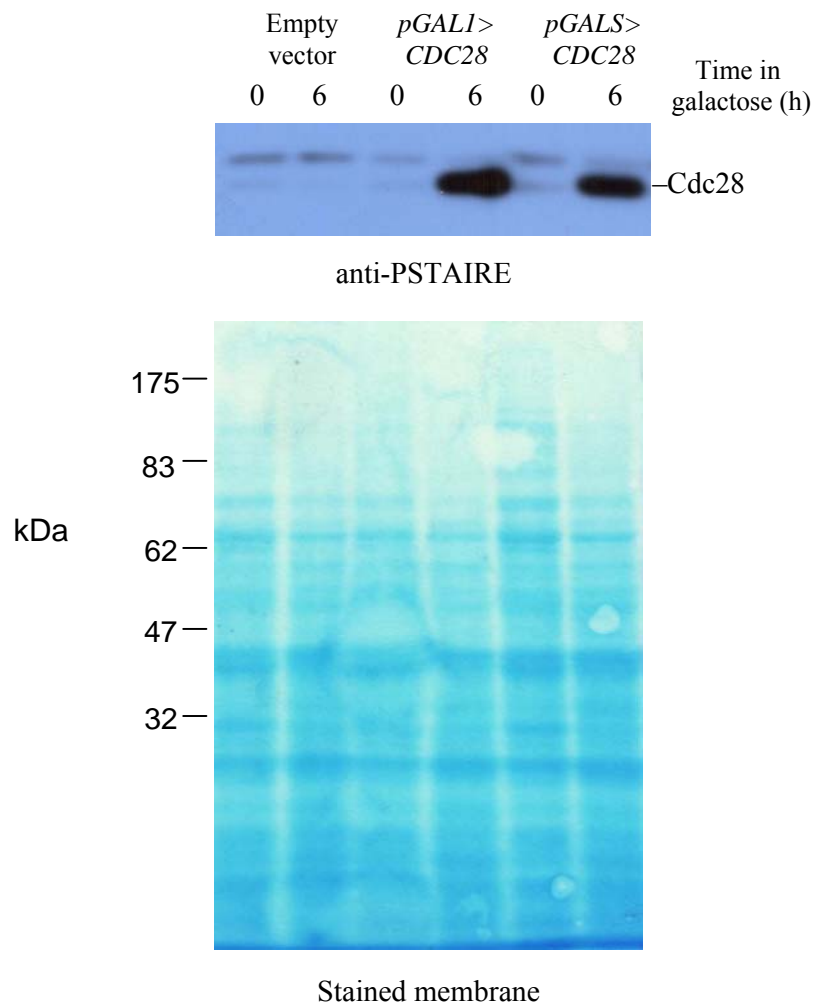


Fig. 12. Overexpression of Cdc28 using the *GAL1* and *GALS* promoters in wild type cells.

Levels of Cdc28 protein were determined by Western blot analysis using an anti-PSTAIR antibody. Immediately after transfer, membranes were stained using the “MemCode” reagent (Pierce) to confirm equal loading and uniform and complete transfer. The molecular weight of Cdc28 is 34 kDa.

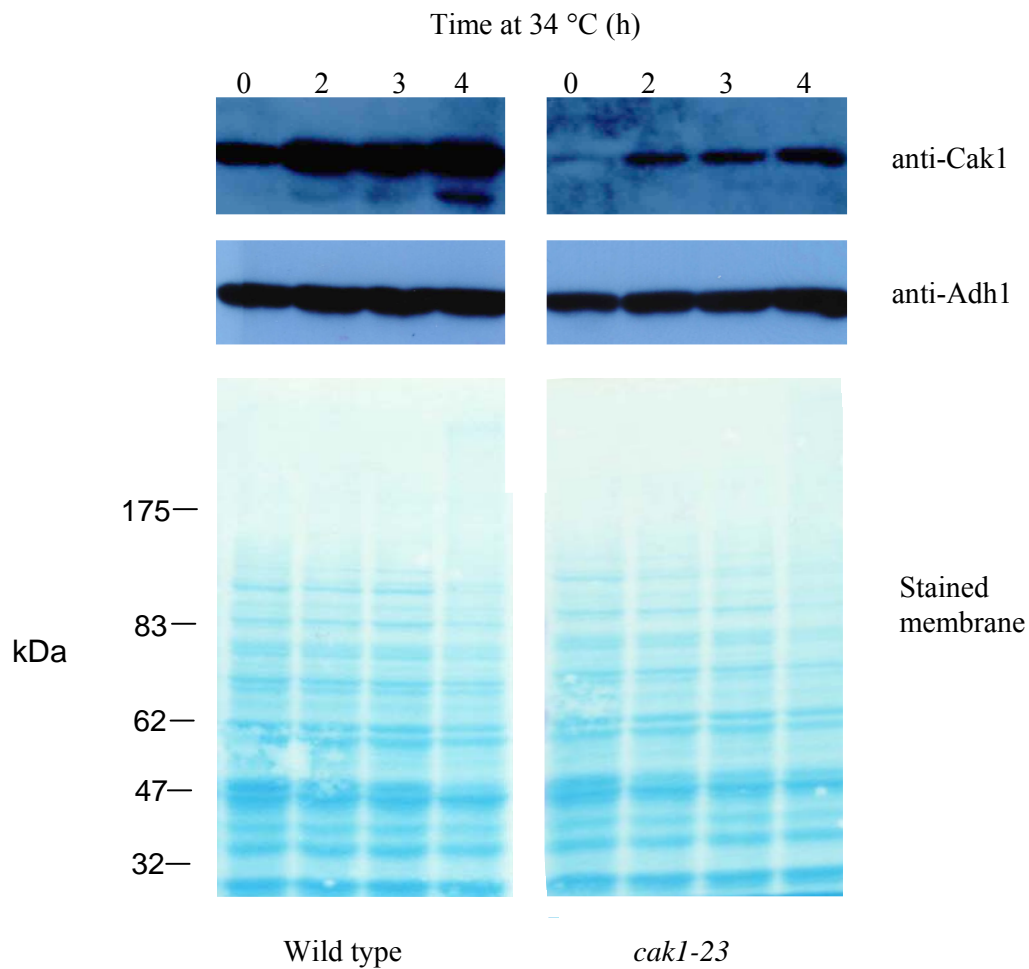


Fig. 13. Lower Cak1 levels in the *cak1-23* mutant at both permissive and restrictive temperatures. Overnight cultures at 25 °C of wild type and *cak1-23* were shifted to 34 °C and aliquots taken at the indicated times. Protein extracts were prepared and loaded onto 10% SDS-PAGE gels. The separated proteins were transferred to nitrocellulose membranes which were stained reversibly first, and then processed for sequential Western analysis with anti-Cak1 and anti-Adh1 antibodies. The molecular weight of Cak1 is 44 kDa and that of Adh1 is 36 kDa.

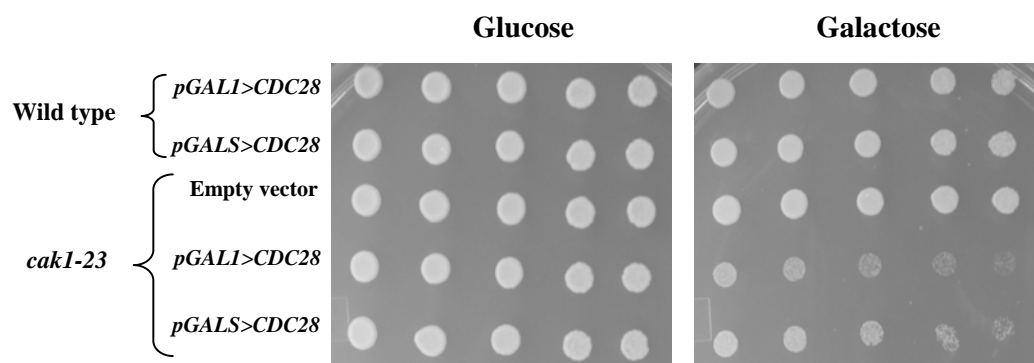


Fig. 14. Overexpression of Cdc28 is deleterious in the *cak1-23* mutant. Wild type and *cak1-23* transformants harboring the indicated plasmids were grown under non-repressive conditions for the *GAL1* and *GALS* promoters, until saturation. The saturated cultures were serially diluted and aliquots from each of the dilutions spotted on selective media containing glucose (promoter OFF) or galactose (promoter ON). Plates were incubated at 25 °C for 2 - 3 days.

with plasmids harboring no insert, *pGALI>CDC28*, or *pGALS>CDC28*. The transformants were grown to saturation in raffinose, serially diluted (2-fold) and 5- μ l aliquots were spotted on either glucose- or galactose- containing media. Plates were incubated at 25 °C. While the effect in wild type cells was minimal, the *cak1-23* mutant was extremely sensitive to overexpression of Cdc28, in a dosage dependent manner (note the lower growth in cells transformed with *pGALI>CDC28*).

We next investigated whether high copy numbers of the non-complementing *cak1*-KD allele suppress the deleterious effect caused by overexpression of Cdc28 in *cak1-23*. We co-transformed *cak1-23* cells with the 2 μ *URA3* plasmid harboring *cak1*-KD, and either empty *LEU2* vector control or *pGALS>CDC28* (Fig. 15). Transformants harboring the indicated plasmids were grown to saturation in raffinose-containing media lacking leucine and uracil. To examine growth rates, saturated cultures were serially diluted (3-fold) and aliquots spotted on the indicated media containing either glucose or galactose. Plates were incubated at 23 °C or 30 °C. We found that increasing copy numbers of *cak1*-KD using the 2 μ plasmid resulted in minor suppression (evident only at the permissive temperature 23 °C). The increase in copy numbers is limited by the replication of the 2 μ plasmid (up to ~60 – 100 copies) and under these conditions, expression of Cak1-KD causes no obvious effect on growth of *cak1-23* (top panel). We suspected that the higher expression levels that can be achieved with the *GAL* promoters (up to ~200 fold) might cause a dominant negative effect. We tested this possibility and as shown in the lower panel, overexpression of *cak1*-KD using the *GALI* promoter shows a clear deleterious effect even at 25 °C.

Therefore, we cannot rule out a compound effect in these cells that prevent the observation of a suppressing effect due to increased numbers of *cak1*-KD in *cak1-23* cells overexpressing Cdc28. Clarification of this issue will need the isolation of *cak1* mutants that are not able to bind to Cdc28,

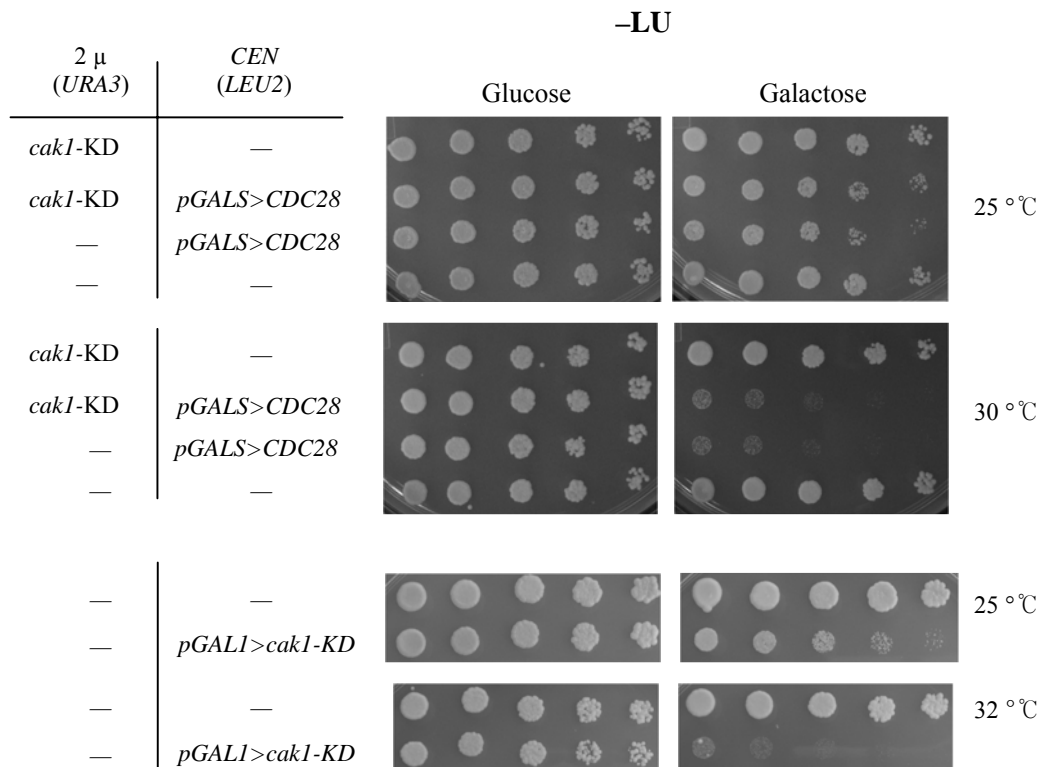


Fig. 15. Compound effect in *cak1-23* overexpressing Cdc28 and/or *cak1*-KD. *cak1-23* cells were transformed with the indicated plasmids and the respective transformants grown in selective media containing raffinose. Saturated cultures were serially diluted and aliquots from each dilution spotted on media lacking leucine and uracil and containing either glucose or galactose. Plates were incubated for 2 – 3 days at the indicated temperatures.

which will allow us to study independently the catalytic and binding roles of Cak1.

Cak1 is subjected to proteolysis when cells cease dividing

Kaldis *et al.* reported that Cak1 is stable in actively growing cells, but becomes unstable when cells enter stationary phase (22). To learn more about how Cak1 levels are regulated, we follow Cak1 protein levels in wild type cells growing in rich media for up to 48 hours. These preliminary studies showed that Cak1 levels decrease dramatically after cells reach an OD_{600nm} >4.0, and Western blot analysis also revealed bands of smaller size that showed increasing intensity as the time course progressed.

To confirm these results, we used wild type cells expressing GST or a GST-Cak1 fusion under the control of the *GALI* promoter (tag at the amino terminus). Cells were grown overnight in selective media containing raffinose, cultures were diluted to OD_{600nm} 0.3, and galactose added to 2% ("0") (Fig. 16). Aliquots were taken at the indicated times. Cak1 levels were determined by Western blotting using both anti-Cak1 and anti-GST antibodies. GST-Cak1 levels clearly show a decrease between the 9 h and 23 h time points. To check the possibility of non-specific proteolysis of Cak1, we used cells lacking *PEP4*, one of the major vacuole proteases (21) and prepare the protein extracts under denaturing conditions. Under these conditions, we observed a decrease in Cak1 protein levels from the 15 hour time point, as growth in both cultures started slowing down and reaching a plateau in growth rates (Fig. 17).

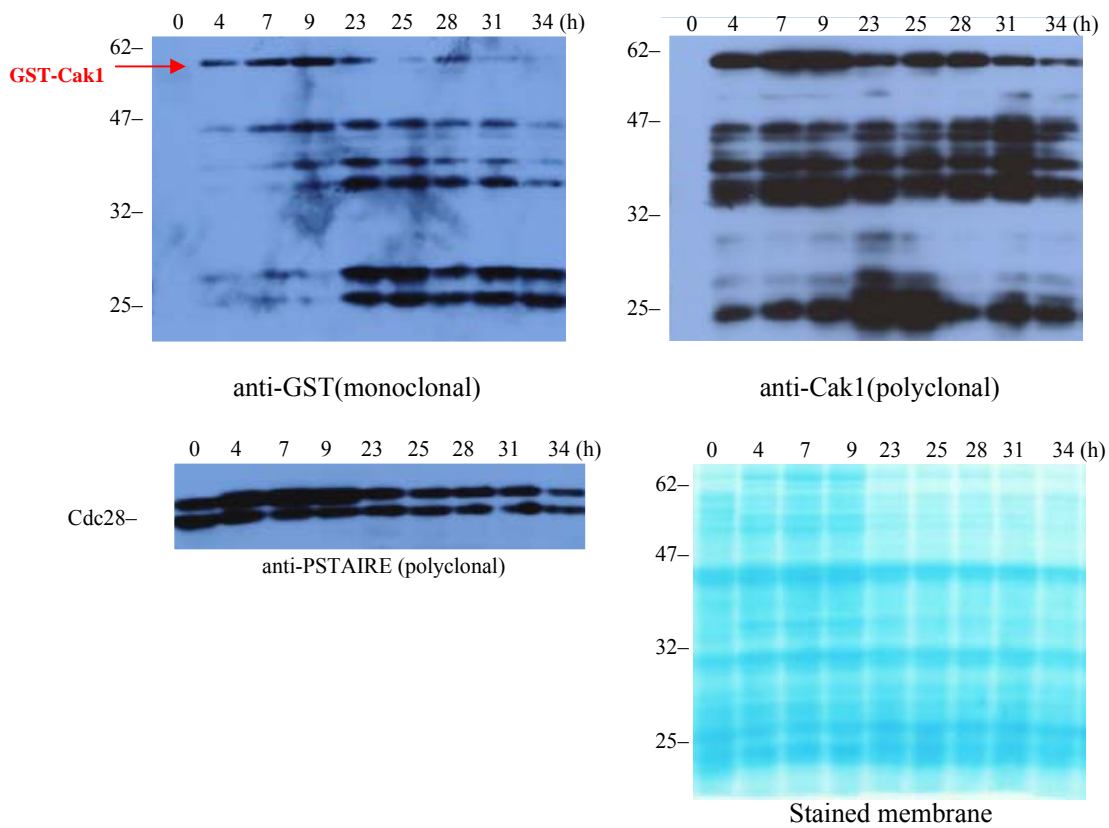


Fig. 16. Cak1 is subjected to proteolytic degradation as cells cease dividing. Aliquots were taken at the indicated times from asynchronous cultures of cells expressing GST-Cak1 under the control of the *GALI* promoter (“0”, time at which galactose was added to start induction of expression). The different panels show the results of sequential Western blot analyses of the same membrane with the indicated antibodies. The molecular weight of GST-Cak1 is 68 kDa but in our system, the fusion protein consistently migrates below our 62 kDa marker (identity confirmed with both monoclonal and polyclonal antibodies).

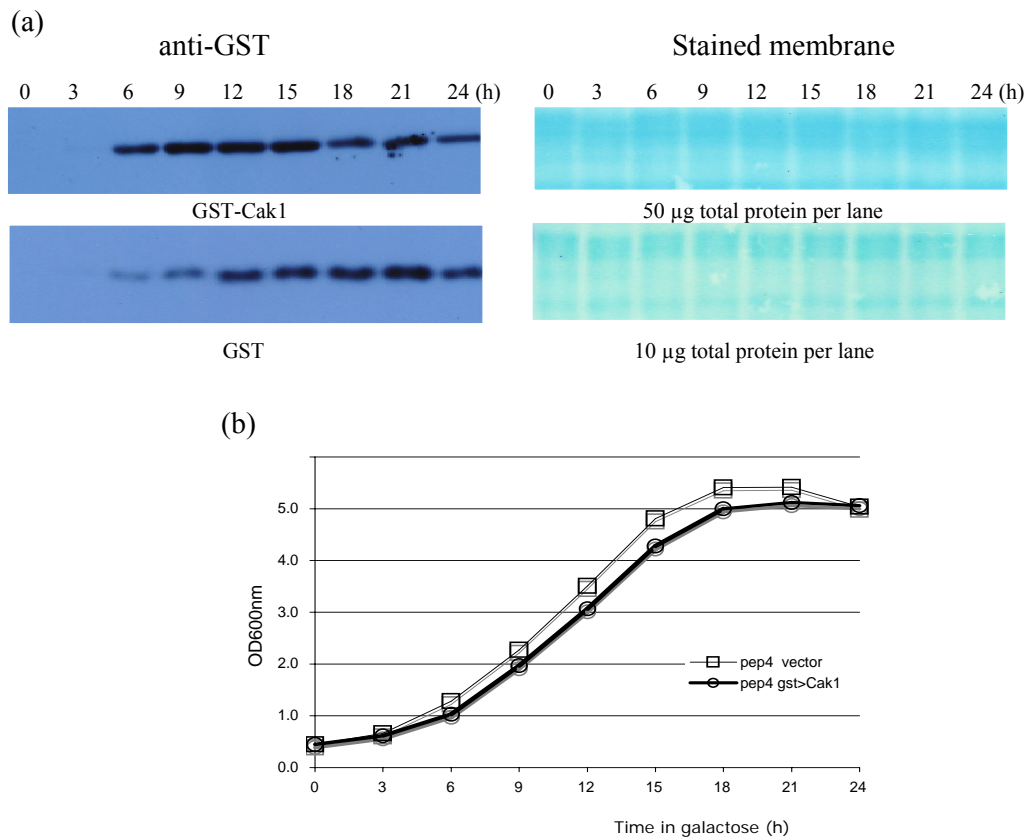


Fig. 17. Cak1 protein levels decrease when cells reach stationary phase.

pep4::kanMX6 cells transformed with empty vector or *pGAL1>GST-CAK1* were grown in selective media containing 2% galactose. Aliquots were taken at the indicated times. (a) Extracts containing 50 or 10 µg of total proteins were separated by SDS-PAGE and analyzed by Western blotting using monoclonal anti-GST antibody. (b) Growth rates were inferred measuring OD_{600nm} at each time point.

CHAPTER IV-Discussion

In previous work, our laboratory identified a number of cyclin-dependent kinase *cdc28* mutants defective in mitotic regulation that accordingly, exhibit sensitivity to microtubule inhibitors and chromosome loss. Several of the mutations were found to localize to the carboxyl terminus of Cdc28 (residues Arg287 and Arg288), which led us to undertake further studies of this region. The carboxyl termini of CDKs are highly conserved but had not been previously implicated in any essential function. Mutations in the last 12 amino acids of Cdc28 were found to cause temperature sensitivity and high chromosome instability. Six *cdc28*^{CST} alleles were isolated and characterized. These data suggested an essential role for the carboxyl terminus of Cdc28 during mitotic progression. Importantly, it was subsequently found that high-copy Cak1 suppressed both the temperature sensitivity and chromosome instability defects in the *cdc28*^{CST} mutants, independently of Cak1's kinase activity.

In recent studies, we found that the *cdc28*^{CST} mutants are extremely dependent on Cak1 dosage and integrity. When *cdc28*^{CST} mutants are transformed with plasmids harboring *cdc28*-T169A, an unphosphorylatable allele, transformation efficiencies dramatically decrease (Fig. 9). Furthermore, the *cdc28*^{CST} mutants become inviable when the wild type Cak1 allele is substituted with one that is temperature sensitive or modified with the addition of carboxy-terminal epitope tags. These results support a model in which Cak1 not only phosphorylates Cdc28 but also interacts with its carboxyl terminus, functioning as an assembly factor that promotes stabilization of the Cdc28 complexes.

Importantly, our results also indicate that actively growing yeast cells require maintenance of an optimum Cdc28:Cak1 ratio. Increasing *CDC28* gene copy numbers by one or two, via transformation with a low copy plasmid harboring *CDC28*, causes low transformation efficiency in wild type cells (Fig. 11). The expression and activity of Cak1 remains constant throughout the mitotic cell cycle;

however, Kaldis *et al.* have found that Cak1 levels decrease when cells enter stationary phase (22). Yeast cells arrest at G1 phase when nutrients, including ammonia, sulfate, and carbon sources are depleted (3). Sustain arrest due to nutrient depletion causes entry into stationary phase, which allows cells to maintain viability for extended periods and resume growth when appropriate nutrients are added (42). Our studies confirmed that GST-Cak1 protein levels decrease when cells cease dividing and enter into stationary phase, using both monoclonal anti-GST and polyclonal anti-Cak1 antibodies (Figs. 16-17). While the particular sensitivities and specificities of the antibodies made the analysis cumbersome (different intensities in some of the bands), analysis with both antibodies revealed a very good correlation between growth rates and GST-Cak1 protein levels.

Heideman *et al.* reported that Cdc28 levels also decrease when nutrients become depleted, but cells rapidly start producing Cdc28 when transferred into rich medium (31). Accordingly, we hypothesize that Cak1 binds to Cdc28 to ensure presence of high CDK activities when cells sense optimum nutrient conditions and are actively growing. When nutrients become depleted and cells reach stationary phase, the stability of Cak1 decreases probably due to a specific proteolytic mechanism. Under these unfavorable conditions for growth, lowering of Cak1 levels results in instability and inactivation of Cdc28 complexes, and stalling of cell-cycle progression.

Working model:

Studies on the respective human homologues have indicated a K_d of 48 nM for the Cdk2-cyclin A binding (19), and 1.5 μ M for that of Cdk2 and Cks1 (35). On the other hand, the laboratories of Erin O'Shea and Jonathan Weissman performed a global analysis of protein localization in yeast that involved the construction of protein fusions with a tandem-affinity purification tag. Their studies

included quantitative Western blot analyses that determine the number of molecules per cell of a large fraction of the yeast proteome, in actively growing cells ([http:// yeastgfp.ucsf.edu](http://yeastgfp.ucsf.edu)) (15). Taking into consideration the binding affinities and copy numbers of each Cdc28, the Cks1 activator, the major mitotic cyclin Clb2, and Cak1, we propose that Cdc28 is sequentially activated as indicated in Fig. 18. Given that Cak1 exhibits kinase activity throughout the cell cycle, residue Thr169 in Cdc28 is constitutively phosphorylated: (I) Cdc28 and Cks1 bind with high affinity and are both present in high copy numbers that remain constant in actively growing cells (6670 and 8780, respectively). (II) Since cyclins are present in significantly lower numbers (339 for Clb2), for short periods of time, and only at specific cell cycle stages, their association to Cdc28~Cks1 occur in a subsequent step. The Cdc28~Cks1~Clb2 complex exhibits high activity but lacks stability. (III) Stabilization of the complex is achieved via Cak1 binding, which ensures maintenance of high CDK activity. (IV) Nutrient depletion forces cells to stop cell-cycle progression and enter stationary phase: Degradation of Cak1 occurs first, and the lowering of Cak1 levels results in instability of the Cdc28 complexes. Because at least Cak1, Cdc28 and cyclins are degraded, this step is irreversible and therefore, *de novo* expression of Cdc28, Cak1, and cyclin subunits are required to resume cell proliferation.

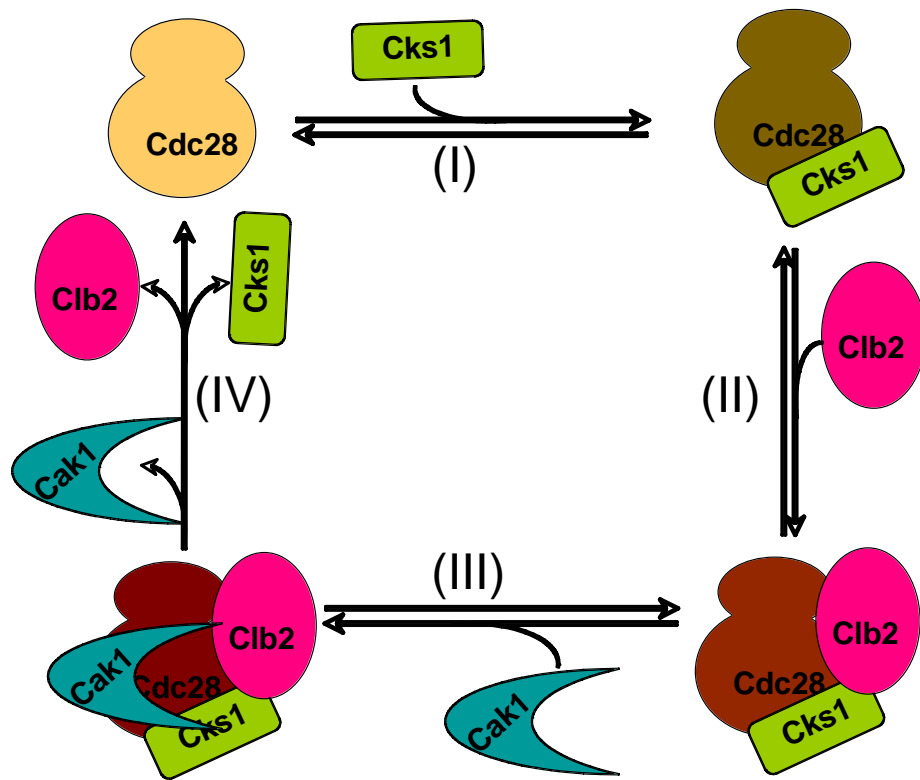


Fig. 18. Working model: In a role that is separated from phosphorylation of Cdc28, Cak1 functions as an assembly factor that maintains Cdc28 complexes stable in actively growing cells.
See text for details.

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