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A phosphorylation-independent role for the yeast cyclin-dependent kinase activating kinase Cak1

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Abstract

Cdc28 is the main cyclin-dependent kinase (CDK) directing the cell cycle in the budding yeast *Saccharomyces cerevisiae*. Besides cyclin binding, Cdc28 requires phosphorylation by the Cak1 kinase to achieve full activity. We have previously isolated carboxy-terminal *cdc28*^{CST} mutants that are temperature sensitive and exhibit high chromosome instability. Both phenotypes are suppressed by high copy Cak1 in a manner that is independent of its catalytic activity and conversely, combination of *cdc28*^{CST} and *cak1* mutations results in synthetic lethality. Altogether, these results suggest that for the Cdc28 complexes to remain stable and active, an interaction with Cak1 is needed via the carboxyl terminus of Cdc28. We report two-hybrid assay data that support this model, and results that indicate that actively growing yeast cells require an optimum Cdc28:Cak1 ratio. While Cak1 is constitutively active and expressed, dividing cells tightly regulate Cak1 protein levels to ensure presence of adequate levels of Cdc28 CDK activity.

Keywords

Cyclin-dependent kinase; Cdc28; Cak1; Yeast; Cell cycle

1. Introduction

The Cdc28 cyclin-dependent kinase (CDK) is the main director of the cell cycle in *Saccharomyces cerevisiae*. To be active, Cdc28 requires association with one of nine cyclins (Cln1–3 and Clb1–6) and the Cks1/Suc1 subunit, and phosphorylation at the activation or T loop (Mendenhall and Hodge, 1998; Harper and Adams, 2001; Murray, 2004; Loog and Morgan, 2005). The activating phosphorylation of CDKs is carried out by the CDK-activating kinase CAK, which comprises a family of monomeric and heterotrimeric enzymes (Kaldis, 1999; Lolli and Johnson, 2005).

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In *S. cerevisiae*, the sole CAK activity is provided by the monomeric enzyme Cak1 (Espinoza et al., 1996; Kaldis et al., 1996; Thuret et al., 1996), which is required for both G1-S and G2-M transitions in vegetative cells (Sutton and Freiman, 1997) and meiosis (Wagner et al., 1997; Schaber et al., 2002; Schindler et al., 2003), and phosphorylates other substrates besides Cdc28 (Espinoza et al., 1996; Kaldis et al., 1996; Chun and Goebl, 1997; Yao and Prelich, 2002; Schindler et al., 2003; Ostapenko and Solomon, 2005). Other monomeric CAK activities have been reported in *S. pombe* (Csk1), *C. albicans* (Cak1), and *A. thaliana* (Cak1At). Interestingly, these enzymes are able to complement *S. cerevisiae cak1* mutants and share several kinetic properties (Tsakraklides and Solomon, 2002).

The heterotrimeric CAK prevails in metazoans and consists of the Cdk7/p40^{MO15} catalytic subunit associated with cyclin H and the assembly factor Mat1 (Poon et al., 1993; Solomon et al., 1993; Fisher, 2005). Besides its role in CDK activation, Cdk7 also functions in basal transcription as part of the transcription factor IIH complex (TFIIH), phosphorylating the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II. Heterotrimeric Cdk7 complex orthologs have been found in most eukaryotes including *S. pombe* (Mcs6/Mcs2/Mcr1) (Hermand et al., 1998; Saiz and Fisher, 2002). The two key CAK functions are separated in *S. cerevisiae*: Cak1 acts predominantly as a Cdc28 activator and cell-cycle regulator, while another Cak1 substrate, the Kin28 CDK acts as the CTD kinase and is the Cdk7 ortholog (Kaldis, 1999). Reciprocally, Kin28 complexes do not phosphorylate Cdc28 (Simon et al., 1986; Cismowski et al., 1995; Espinoza et al., 1998). Other Cak1 substrates such as the Ctk1 and Bur1 CDKs also function as CTD kinases (Espinoza et al., 1998; Kaldis, 1999; Murray et al., 2001; Yao and Prelich, 2002; Ostapenko and Solomon, 2005).

Functional interactions have been reported between *CAK1* (via *CTK1* and *BUR1*) and *SSU72* (CTD phosphatase) and some components of the PAF transcriptional elongation complex, which also promotes histone modifications and mRNA processing (Ganem et al., 2006). More recently, the *cak1*-P212S allele was isolated by virtue of its hyperpolarized growth and synthetic lethality with a mutation in one of the chitin synthase genes (Schmidt et al., 2008). Likewise, Cak1 and Cdc28 have been shown to participate in the regulation of sister-chromatid cohesion in S phase (Brands and Skibbens, 2008). Despite its several functions, the essential role of Cak1 relates to the activating phosphorylation of Cdc28. This was demonstrated by the isolation of *cdc28* mutants that remain viable in the absence of Cak1 (Levine et al., 1998; Cross and Levine, 2000).

While a great deal is known about CDKs at the structural and mechanistic levels, no specific function has been assigned to their carboxyl termini. Interestingly, the last eight amino acids in the human Cdk2 enzyme locate to almost opposite positions when bound to cyclin A2 or cyclin E1, but the implications of these different conformations remain unknown (Honda et al., 2005). Further, our previous results indicate that the carboxyl terminus of Cdc28 is essential for proper mitotic progression since mutations in this region induce chromosome instability and high lethality at rising temperatures (*cdc28*^{CST} mutants) (Kitazono et al., 2003). High copy Cak1 suppresses both phenotypes and interestingly, suppression of the temperature sensitivity is independent of the catalytic activity of Cak1. This result suggested that the defects in the *cdc28*^{CST} mutants are not simply due to diminished phosphorylation by Cak1 (Kitazono and Kron, 2002). Instead, we favor a model in which suppression is due

to enhanced Cak1 binding to Cdc28. Here, we report studies that demonstrate a key role for the carboxyl terminus of Cdc28 in interaction with Cak1. We also found that maintaining an optimum Cdc28:Cak1 ratio is crucial to sustain active growth. Based on these results, we suggest that actively growing cells rely on Cak1 not only to phosphorylate but also to stabilize Cdc28 complexes.

2. Materials and methods

2.1. Plasmids, strains, and general yeast methods

All basic procedures were as described previously (Kitazono and Kron, 2002), using cells of the W303 genetic background (Thomas and Rothstein, 1989). The strains used in this study are shown in Table 1, and the plasmids in Table 2.

Plasmids p*GAL1>CDC28* and p*GALS>CDC28*, and p*GAL1>CAK1* were constructed by amplifying the respective sequences using pFA6a-kanMX6-p*GAL1* and genomic DNA as templates. The two PCR fragments were then joined together via their overlapping 25 bp ends using the outmost forward and reverse primers. The final fusion-PCR products carried the respective promoter and ORF and flanking restriction enzyme recognition sites for cloning into pRS315 (*CEN LEU2*, *CDC28* plasmids) or pRS316 (*CEN URA3*, *CAK1* plasmids) (Sikorski and Hieter, 1989).

2.2. Two-hybrid assays

We used the system developed by James et al., which is based on the DNA-binding and activation domains of the Gal4 transcription factor (James et al., 1996). Transformants were grown under selective conditions for 4 days at room temperature to obtain saturated cultures and ensure similar cell densities. These cultures were serially diluted (5-fold) and 5 μl aliquots from each dilution spotted on the indicated plates. The densities of the respective spots were compared using ImageJ 1.40g software to determine their mean-gray values. The densities of three consecutive dilutions were determined for each sample and compared to those of the respective control.

2.3. Determination of transformation efficiencies

Wild type, *cdc28-cst2*, and *cdc28-cst8* cells were first transformed with pRS202 (2 μm *URA3*) plasmids harboring no insert, *CAK1*, or *cak1*-KD. Single colonies were obtained, grown under selective conditions, and transformed using suspensions of identical cell densities and amounts of the *CEN TRP1* plasmids carrying *CDC28*-HA or *cdc28*-T169A-HA. After transformation and brief sonication to disrupt aggregates, the cell densities of each of the mixes 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 only uracil, or both uracil and tryptophan. Plates were incubated for 2 to 4 days at 25 °C.

2.4. Protein extract preparations and Western blotting

Cells were collected by centrifugation, washed twice with 1×PBS 10% glycerol (PBSgly) and kept at −80 °C until use. For the experiments described in Fig. 3B, PBSgly contained 8

M Urea. Cells were disrupted in the same buffer used in the washes, containing 1 mM phenylmethane sulfonylfluoride and 1× 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 ADV01 reagent from Cytoskeleton.

Aliquots containing 100 μg of total proteins were loaded onto SDS-PAGE gels and the separated proteins were transferred to nitrocellulose membranes. Transfer efficiency and equal loading were surveyed by staining the membranes using the MemCode kit from Pierce, and/or using anti-Adh1 (1:20000, Calbiochem) or anti-tubulin (1:500, Cytoskeleton) antibody. Cak1 was detected using polyclonal or monoclonal anti-Cak1 antibodies (1:4000), generously provided by P. Kaldis. Cdc28 was detected using a polyclonal anti-PSTAIRE antibody (1:2500, Santa Cruz), which also recognizes the Pho85 CDK (top band in Fig. 7A). Horseradish peroxidase-linked goat anti-mouse, anti-sheep, or anti-rabbit IgG antibody and Super-Signal Substrate (Pierce) were used for detection. The densities of the respective bands were analyzed using the “Image analysis” feature in Adobe Photoshop CS3.

3. Results

3.1. High copy numbers of CAK1 suppress the aberrant morphology of the cdc28^{CST} mutants

Strains harboring mutations in the carboxyl terminus of Cdc28 (*cdc28-cst2*, *-cst3* and *-cst8*, Table 1) are temperature sensitive and exhibit high chromosome instability, phenotypes that are suppressed by high copy numbers of catalytically active or inactive Cak1 (Kitazono and Kron, 2002). The suppression extends to the aberrant morphologies that the *cdc28*^{CST} mutants display even when grown at permissive temperatures (Fig. 1). For this analysis, the respective transformants were grown in media lacking uracil to select for the empty vector control (pRS202, 2 μm *URA3*) or the same plasmid harboring either the wild type or catalytically deficient *CAK1* allele. To confirm that the effects were specific to presence of high copy numbers of *CAK1* or *cak1*-KD, the same transformants were grown in media containing 5-fluoroorotic acid (FOA) to counterselect for the pRS202 plasmids. In all cases, eviction of the plasmids resulted in larger and elongated cells, morphologies that are characteristic of the *cdc28*^{CST} mutants.

3.2. Mutations in the carboxyl terminus of Cdc28 affect interaction with Cak1

Because the suppression was independent on its catalytic activity, we hypothesized that the effect of increasing Cak1 copy numbers on the *cdc28*^{CST} mutants was due to restored Cdc28-cst–Cak1 interaction rather than activating phosphorylation. To investigate this possibility, we first compared the interactions between Cak1 and wild type or mutant Cdc28, using a two-hybrid system (James et al., 1996). Wild type *CAK1* and *CDC28*, and the *cdc28*^{CST} alleles were cloned in the bait (DNA-binding domain of Gal4, DBD, *URA3*) and prey (activation domain of Gal4, AD, *LEU2*) plasmids. Transformants harboring the respective plasmid combinations were grown to saturation, and the cultures serially diluted and spotted onto the indicated plates: Synthetic complete medium lacking uracil and leucine was used to select for presence of the plasmids (−UL); and medium lacking uracil, leucine

and histidine and supplemented with 2 mM 3-aminotriazole (3AT), to select for expression of the *HIS3* reporter gene (Fig. 2). The strain harboring the wild type *CDC28* and *CAK1* plasmids exhibits robust growth on the 3AT plates, indicating a strong interaction between the respective fusion proteins. Plasmids harboring the *cdc28-cst2* and *cdc28-cst8* alleles confer minimal growth on the same media suggesting in each case, a significant loss of interaction with Cak1.

3.3. Cak1 binding to Cdc28 is required for stabilization of Cdc28–Cks1 complexes

Using the two-hybrid system described in Fig. 2, we found that the carboxy-terminal mutations in Cdc28 also affected interactions with the Cks1 activator. To test if Cak1 binding to Cdc28 is required to stabilize the complexes, the two-hybrid assays were performed upon introduction of a high copy plasmid (*TRP1*) harboring no insert, wild type *CAK1*, or catalytically inactive *cak1*-KD (Fig. 3A). Presence of either plasmid causes no evident difference when testing interactions between Cks1 and wild type Cdc28, since all transformants exhibit comparable growth on medium lacking histidine or adenine. On the other hand, increasing the copy numbers of *CAK1* or *cak1*-KD improves the interaction between Cdc28-cst8 and Cks1. This is evident by the 1.3 to 1.6-fold increase in the number of colonies formed in the transformants harboring a *CAK1* or *cak1*-KD plasmid, when compared with the empty plasmid control. Similar results were obtained when testing the effects on the Cdc28-cst2–Cks1 interaction (not shown).

To verify that the levels of the Cdc28-cst8 protein fusions were not affected by overexpression of Cak1, we performed Western blot analyses of extracts from transformants carrying the respective two-hybrid plasmids and the high copy *TRP1* plasmid harboring no insert or *CAK1* (Fig. 3B). While the levels of the DBD-Cdc28-cst8 protein were 20–30% lower than those of the wild type Cdc28 fusion, no difference was observed in the presence of high copy numbers of Cak1. Altogether, these results indicate that in a function that is independent of its catalytic activity, Cak1 binding to Cdc28 is required for stable association with the Cks1 activator.

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

Given that increasing dosage of *CAK1* suppresses both the temperature sensitivity and chromosome instability of the *cdc28*^{cst} mutants (Kitazono and Kron, 2002), we hypothesized that disrupting Cak1 function would be deleterious in that background. First, we tested for genetic interactions between the *cdc28*^{CST} and *cak1–4*, *cak1–17*, and *cak1–23* mutations (Thuret et al., 1996; Wagner et al., 1997; Espinoza et al., 1998). No haploids harboring both mutations could be obtained after multiple attempts, suggesting synthetic lethality. To confirm this, we analyzed meiotic segregants of heterozygous diploids carrying a *CDC28*-pRS316 [*CEN URA3*] plasmid (Fig. 4). In all cases, the segregants harboring both *cdc28*^{CST} and *cak1* mutations remain viable only in the presence of the complementing plasmid. Likewise, the double mutants carrying *CDC28*-pRS316 were unable to form colonies on media containing FOA.

The *cdc28*^{cst} mutants are also non-viable when transformed with plasmids harboring *cdc28*-T169A, in which the residue that is phosphorylated by Cak1, Thr169 has been substituted by

Ala (Fig. 5A). For these experiments, we compared the transformation efficiencies of wild type and the *cdc28*^{CST} mutant strains with empty vector, or plasmids harboring *cdc28*-T169A or wild type *CDC28*. All these are centromeric plasmids that usually exhibit 1–2 copies per cell, but may increase ~12-fold under particular conditions such as presence of the *his3*-Δ*4* allele (Bitoun and Zamir, 1986). No significant effect was observed in the wild type strain transformed with either plasmid (see below), or when the *cdc28*^{cst} mutants were transformed with the empty vector control or wild type *CDC28* plasmid. However, transformation efficiencies considerably decreased when the mutants were transformed with the plasmid carrying *cdc28*-T169A, where formation of a few small colonies is evident only after >1-week incubation (similar results obtained for *cdc28-cst2* and *cdc28-cst3*, not shown).

One explanation for these results is 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 test this possibility, we repeated the assay in the presence of high copy numbers of *CAK1* or *cak1*-KD (Fig. 5B). First, *cdc28-cst8* was transformed with high copy *URA3* plasmids carrying no insert ("—"), *CAK1* or *cak1*-KD. Selected transformants were then subjected to a second transformation with *CEN TRP1* plasmids carrying *CDC28* or *cdc28*-T169A, under identical conditions (cell number and amount of DNA). The final transformation mixes were normalized for cell densities, serially diluted (5-fold), and the respective aliquots spotted on media lacking uracil (−U) or both uracil and tryptophan (−UW). The number of colonies formed in the −U plates were all similar, indicating presence of equal number of cells in the respective dilutions. On the −UW plates, we observed robust growth of transformants with the wild type *CDC28* plasmid. Confirming our previous result (Fig. 5A), hardly any growth was observed in cells transformed with both the empty high copy and the *cdc28*-T169A plasmids. Confirming that the deleterious effect was due to Cak1 titration, presence of high copy numbers of *CAK1* or *cak1-KD* improved the transformation efficiencies, suggesting again that the catalytic activity of *CAK1* was not required for the effect. Similar results were obtained with *cdc28-cst2* (not shown).

3.5. Maintenance of an optimum Cdc28:Cak1 ratio is crucial for growth of wild type cells

In the course of these studies, we noticed that wild type cells consistently exhibited low transformation efficiencies with plasmids harboring either *CDC28* or *cdc28*-T169A. These transformations yielded ~2 to 3-fold lower number of colonies than when using empty vector controls (Fig. 6). Furthermore, we found that the lower transformation efficiency was suppressed by presence of high copy numbers of *CAK1* or *cak1*-KD. We interpret these results as to mean that wild type cells require maintenance of an optimum Cdc28:Cak1 ratio to support active cell divisions. According to this model, alteration of this ratio by increasing the copy numbers of Cdc28 results in formation of unstable or inactive complexes (due to limited levels of Cak1) that titrate out activators such as cyclins and Cks1. Note that while this deleterious effect is significant and clear when assessing transformation efficiencies as shown in Fig. 6, it is subtle and easily unnoticeable when doing transformations "in bulk" (Fig. 5A, see below and Discussion).

In order to test this model, we investigated the effects of increasing Cdc28 levels on growth rates, using plasmids that allow expression of *CDC28* under the control of the *GAL1* promoter and its weaker derivative *GALS* (Fig. 7A) (West et al., 1984; Mumberg et al., 1994). Besides wild type cells, we also tested the *cak1-23* mutant, which exhibits significant lower Cak1 protein levels than the wild type strain, at both permissive and restrictive temperatures (Fig. 7B). We followed growth of population of cells on galactose-containing plates and noticed that the effects on wild type cells were under these conditions, minimal. However, *cak1-23* cells were sensitive to overexpression of Cdc28 even at permissive temperatures, in a dosage-dependent manner (Fig. 7C).

To test if under these conditions increasing copy numbers of non-complementing *cak1*-KD suppresses this deleterious effect, we co-transformed *cak1–23* cells with both the 2 μm plasmid harboring *cak1*-KD and P*gals*>*CDC28*, or combinations with the respective empty vectors. We compared growth rates in plates containing either glucose or galactose at 25 °C (Fig. 7D). Presence of high copy *cak1*-KD allows ~50% increase in growth of cells overexpressing *CDC28*, but the effect was minimal at higher temperatures (not shown). These results suggested the possibility that overexpression of *cak1*-KD had a dominant negative effect in the *cak1–23* mutant. Later studies demonstrated that expression of *cak1*-KD at higher levels using the *GAL1* promoter is deleterious in *cak1–23* and other *cak1* mutants (not shown). On this basis, we conclude that increasing *cak1*-KD copy numbers helps counteracting the deleterious effect of *CDC28* overexpression in *cak1–23* cells up to a threshold above which, the dominant negative is manifested.

4. Discussion

Early studies in yeast have demonstrated that Cak1 phosphorylates monomeric Cdc28 in a step that precedes cyclin binding (Ross et al., 2000). In this scenario, phosphorylation of Cdc28 would require only transient interaction with its T loop. However, Cak1 has been found to co-immunoprecipitate and co-purify with Cdc28 suggesting a stable association (Thuret et al., 1996). On the other hand, studies in a human cell line have determined that CAK activity is needed to stabilize formation of Cdk1-cyclin B complexes but not those that include Cdk2 (Larochelle et al., 2007). Similar findings have been reported for other Cdk1 complexes as well (Desai et al., 1995; Larochelle et al., 1998). Interestingly, our results indicate that the stabilizing effects of Cak1 on Cdc28 are independent of the catalytic activity of Cak1 and therefore, phosphorylation of Thr169. It is possible that this novel role for Cak1 constitutes a mechanism that is exclusive to yeast cells, which need to rapidly adapt to changes in environmental conditions. We suggest that Cak1 has a catalytically independent function that is essential for maintaining active Cdc28 complexes. Mutations in the carboxyl terminus of Cdc28 prevent efficient Cdc28–Cak1 interaction, which in turn affects association with Cks1 (Fig. 3). In this regard, it is important to note that the *cdc28*^{CST} mutants are not suppressed by high copy numbers of Cks1 or cyclin Cln1, suggesting the defects are specific to Cak1 binding (Kitazono and Kron, 2002).

The reported results suggest that actively growing wild type cells require an optimum Cdc28:Cak1 ratio, which cannot be altered by increasing *CDC28* copy number even by one or two (transformation with a *CEN* plasmid harboring *CDC28*, Fig. 6). In yeast, the effects

due to overexpression are usually studied by cloning the respective genes in high copy 2 μm vectors (>10 to 40-fold increase), or by expressing them under the control of strong inducible promoters (diverse increase, usually >20-fold). In this regard, Kitano and colleagues developed a method termed “genetic tug-of-war” that allows determination of upper limit copy numbers without stringent selective mechanisms (Moriya et al., 2006). The authors applied this method to determine the highest permissible copy numbers of 30 genes involved in cell-cycle control. Several genes coding for Cdc28 regulators were found to accumulate only to low copy numbers in their system (*CDC14* phosphatase, *SIC1* inhibitor, *CLB5,3,2* cyclins, *SWE1* kinase). In contrast to our findings (Fig. 6), no such significant effect was reported for *CDC28*, which coincides with other reports stating that overexpression of *CDC28* has no effect on growth rates (Nacht et al., 1995). However, it is important to note that all these studies were performed in cells that had grown for several generations with high copy numbers of Cdc28 (at least >10 to 20-fold considering the copy number of 2 μm vectors). Therefore, one cannot rule out that these cells had gone through rounds of selection for those able to tolerate the increase in Cdc28 dosage, had adapted, and/or acquired extragenic suppressor(s). This and other systems lack the sensitivity that we have accomplished by measuring the transformation efficiencies of the different low copy *CEN* plasmids tested (Figs. 5 and 6).

While Cak1 expression and activity remain unchanged throughout the yeast cell cycle, Kaldis et al. found a significant drop in Cak1 protein levels as cells reach stationary phase (Kaldis et al., 1998). Likewise, Cdc28 levels decrease to <78% as cells cease dividing due to nutrient depletion (Wittenberg and Reed, 1988) and conversely, addition of glucose to these cells induces immediate production of Cdc28 (Newcomb et al., 2003). Interestingly, other studies have demonstrated that cultured cells from neoplastic tissues exhibit higher Cdk1 levels than those derived from normal tissues (Gannon et al., 1998). Altogether, these studies suggest that there is a close correlation between growth rates and CDK levels and activities. In this regard, we propose that in yeast cells, Cak1 has a key role promoting maintenance of optimum Cdc28 levels and activities. A drop in Cak1 levels turns Cdc28 inactive and/or unstable, to ensure that cells remain in stationary phase until nutrients are replenished. We have found conditions in which this scenario is reenacted: one involves mutations at the carboxyl terminus of Cdc28 that render it unable to efficiently interact with Cak1. The lack of interaction affects Cdc28 to such an extent that the *cdc28*^{CST} mutants exhibit severe temperature sensitivity and unregulated mitotic progression. Two other conditions involve alteration of the Cdc28:Cak1 ratio via introduction of an additional copy of *CDC28* in a *CEN* plasmid (Fig. 6), and overexpression of Cdc28 in *cak1-23* cells (Fig. 7C and D).

Cak1’s essential function is to phosphorylate Cdc28, which was demonstrated via the isolation of *cdc28* mutants harboring several substitutions including Thr169Glu that allow growth in the absence of *CAK1* (Cross and Levine, 1998; Cross and Levine, 2000). These studies also established that rounds of phosphorylation and dephosphorylation of Thr169 are not required during the yeast cell cycle. Since the introduction of several substitutions in Cdc28 turns Cak1 dispensable, the authors discussed three possible mechanisms to explain its evolutionary persistence: Existence of some yet unidentified regulation of Cdc28 that is

dependent on phosphorylation by Cak1; that the phosphorylation most effectively colludes with cyclin binding to activate Cdc28; or that there is absolute need to phosphorylate Thr169 and provide a dianionic group to induce the required conformational changes in Cdc28. Our results add one more explanation for the persistence of Cak1 in yeast cells: Functioning as an assembly factor for Cdc28 complexes, Cak1 helps ensure maintenance of high CDK activity in actively growing cells.

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Abbreviations

CDK	cyclin-dependent kinase
cdc28^{CST}	cdc28 mutant deficient in maintenance of chromosome stability
CTD	carboxy-terminal domain of the large subunit of RNA polymerase II
FOA	5-fluoroorotic acid
DBD	DNA-binding domain
AD	activation domain

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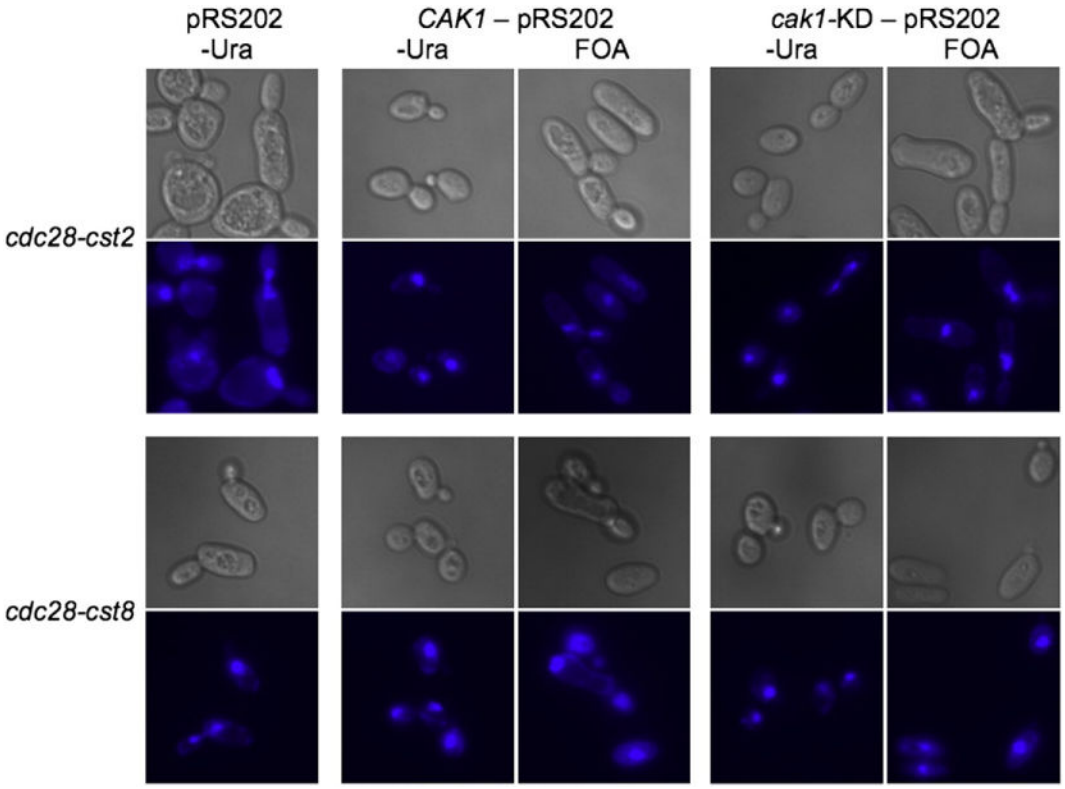

Fig. 1.

High copy numbers of catalytically active or inactive Cak1 suppress the aberrant morphologies of the *cdc28*^{CST} mutants. Cells grown in media lacking uracil (−Ura) or in rich media (after FOA treatment), were fixed with 70% ethanol, and resuspended in 70% glycerol containing 2.5% of the anti-fading agent DABCO and 1 μg/ml 4′,6-Diamidino-2-phenylindole (DAPI). DIC and fluorescence images were obtained using an AxioObserver Zeiss inverted microscope, a Plan-Apochromat 100×/1.4 oil objective, and an Orca ER1394 camera.

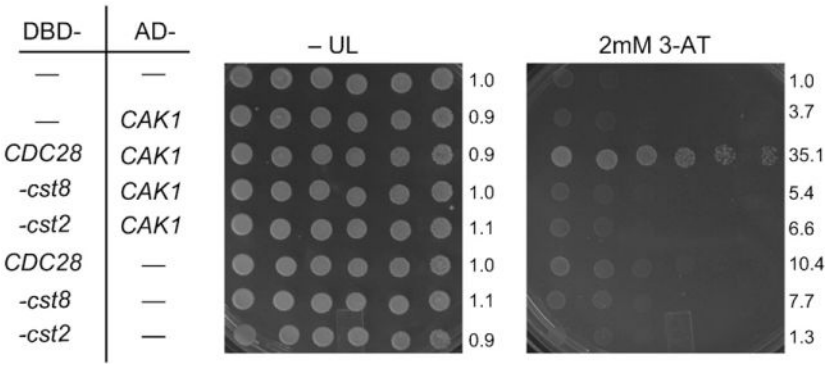

Fig. 2.

Mutations in the carboxyl terminus of Cdc28 affect its interaction with Cak1. The two-hybrid tester strain developed by James et al. was transformed with the bait ("DBD", Gal4 DNA-binding domain, *URA3*) and prey ("AD", Gal4 activation domain, *LEU2*) plasmids harboring no insert ("—"), wild type *CAK1* or *CDC28*, or the *cdc28-cst2* or *cdc28-cst8* alleles, as indicated. The transformants were grown to saturation under selective conditions, and the respective cultures serially diluted (5-fold) and spotted onto the indicated plates. "−UL" medium lacks uracil and leucine; "2 mM 3-AT" medium lacks uracil, leucine and histidine, and contains 2 mM 3-aminotriazole to select for the activation of the *HIS3* reporter gene. The numbers in the figure were obtained by comparing the mean-gray values of the spots using ImageJ software. The densities of three consecutive dilutions of each of the transformants were measured and compared to those from the empty vector control.

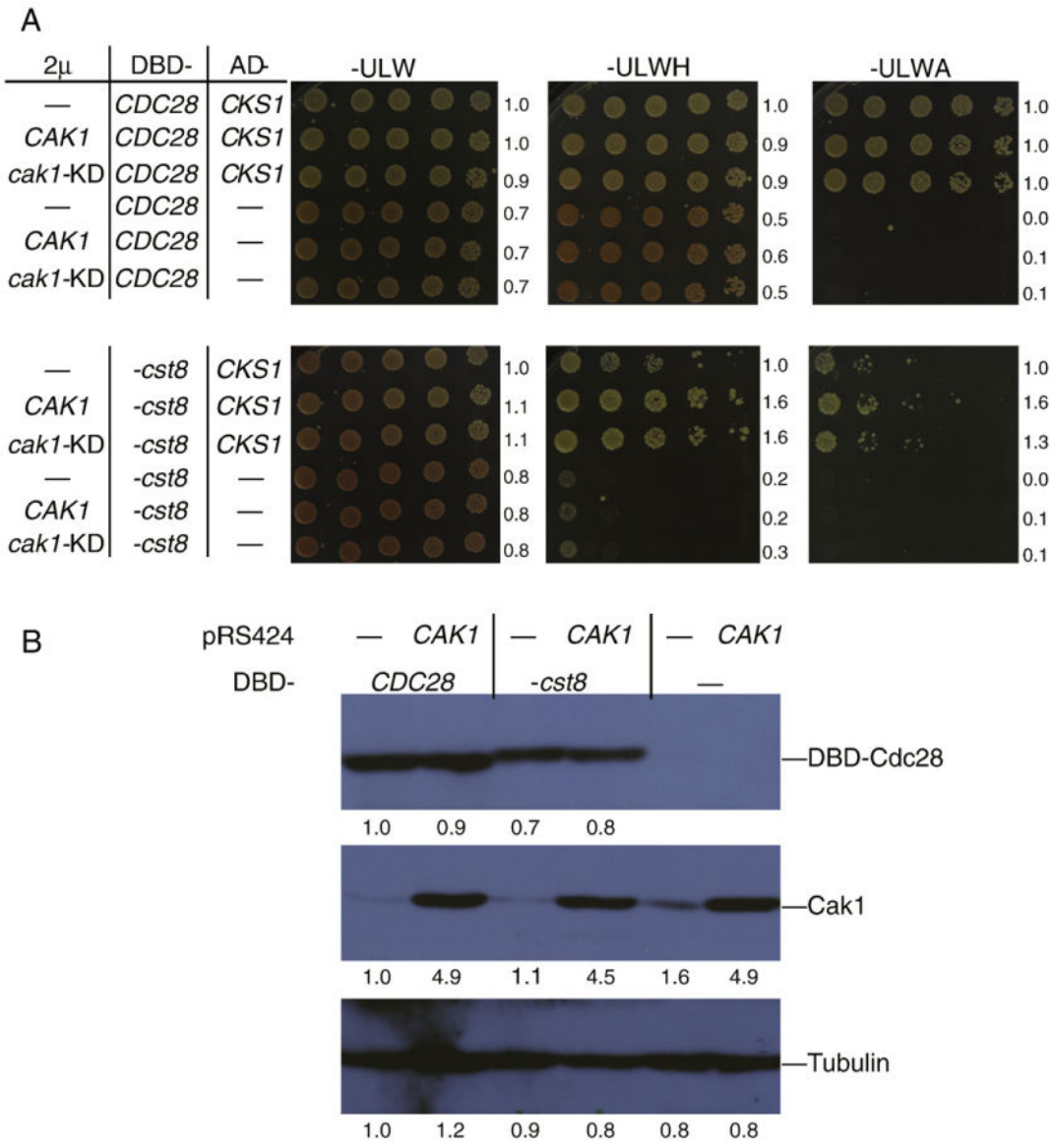

Fig. 3.

Increasing levels of Cak1 (catalytically active or inactive) promote stronger two-hybrid interaction between Cdc28-cst8 and Cks1. (A) The same system and procedures described in Fig. 2 were used for these studies, except that a third plasmid (2 μm *TRP1*) harboring no insert, *CAK1*, or *cak1*-KD was introduced. "−ULW" lacks uracil, leucine and tryptophan. The two other plates allow selection for the activation of the *HIS3* and *ADE2* reporter genes: "5 mM 3-AT" is −ULWH medium that lacks uracil, leucine, tryptophan, and histidine; and includes 5 mM 3-aminotriazole. "−ULWA" lacks uracil, leucine, tryptophan, and adenine. (B) Overexpression of Cak1 has no effect on the stability of the Cdc28-cst8 protein. The two-hybrid tester strain was transformed with the bait plasmid harboring no insert, *CDC28* or *cdc28-cst8*, empty prey plasmid, and the 2 μm *TRP1* vector pRS424 harboring no insert or *CAK1*. Aliquots containing 100 μg of total proteins were loaded onto 10% SDS-PAGE gels. The separated proteins were blotted onto nitrocellulose membranes, which were then

sequentially treated with the following antibodies: rabbit polyclonal anti-PSTAIRE (Cdc28), mouse monoclonal anti-Cak1, and sheep polyclonal anti-tubulin. The relative densities of the bands were analyzed for the Cdc28 and Cak1 blots, and are indicated below the respective blots.

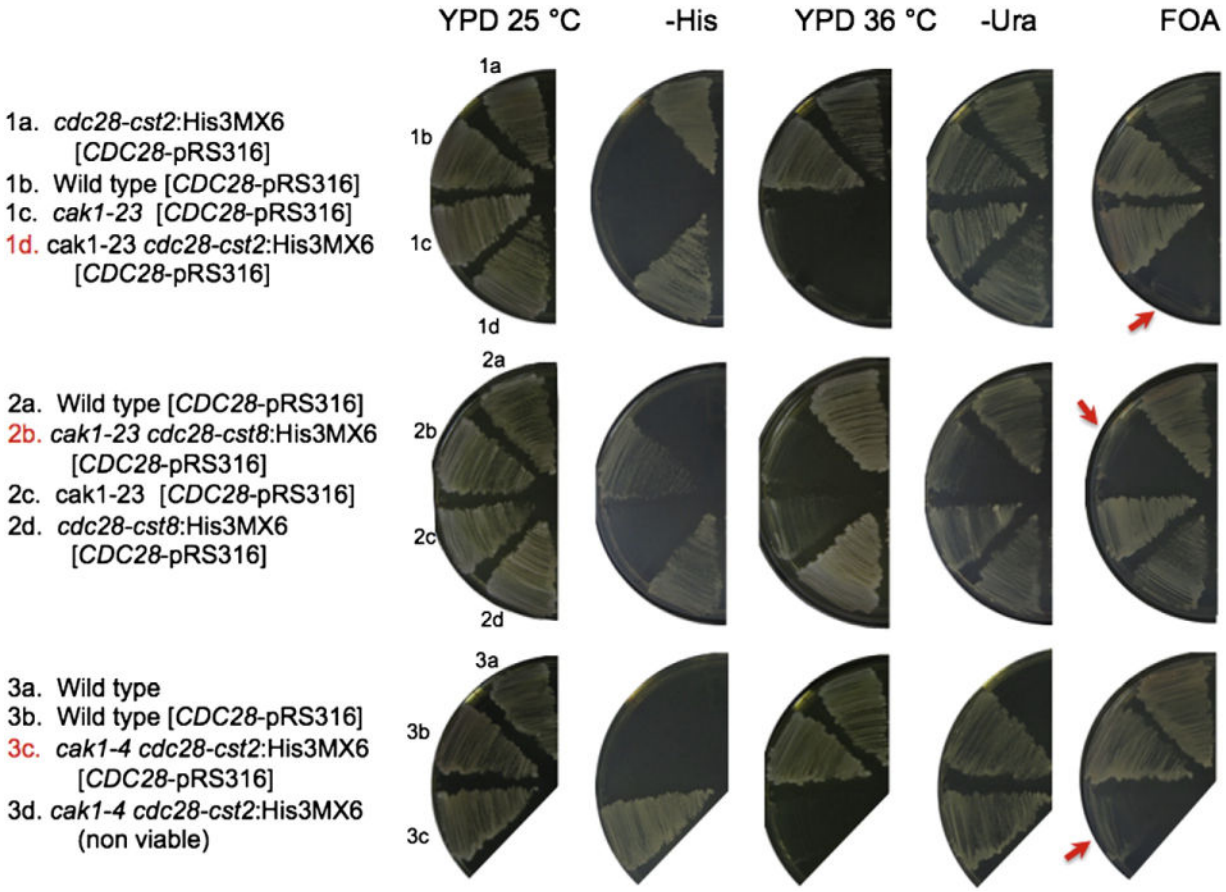

Fig. 4.

cdc28^{CST} and *cak1* mutations are synthetic lethal. Three different heterozygous mutants were transformed with a *CEN URA3* plasmid harboring *CDC28* and sporulated. Selected meiotic segregants were analyzed for growth in the indicated media and conditions. Lack of growth on FOA-containing media (indicated by the arrows) confirmed lack of viability of the *cdc28*^{CST} *cak1* double mutants. Similar results were obtained when testing all possible combinations of the *cdc28-cst2*, *cdc28-cst8* and *cak1–23* and *cak1–4* mutations.

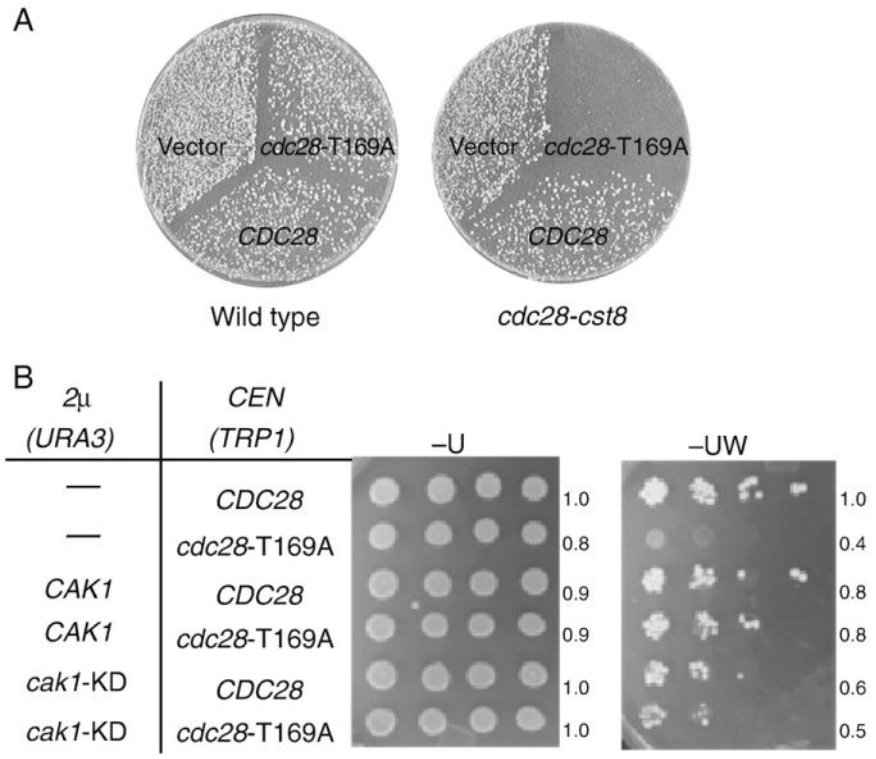

Fig. 5.

Plasmid harboring *cdc28*-T169A shows reduced transformation efficiency in *cdc28*^{CST} mutants. (A) Wild type and *cdc28-cst8* were transformed under identical conditions, with either empty vector or the same vector harboring wild type *CDC28* or the unphosphorylatable allele *cdc28*-T169A. Aliquots from each transformation mix were spread on medium lacking tryptophan and the plates were incubated at 25 °C. Pictures were taken after 3–4 days. (B) *cdc28-cst8* was sequentially transformed with the indicated 2 μm (*URA3*) and *CEN* (*TRP1*) plasmids. Transformation with the latter was carried out under identical conditions (cell densities of cultures, amount of plasmid DNA). Transformation mixes with the *CEN TRP1* plasmids were serially diluted 5-fold, and aliquots spotted on medium lacking only uracil or both uracil and tryptophan. "—" indicates vector control (no insert). Plates were incubated at 25 °C.

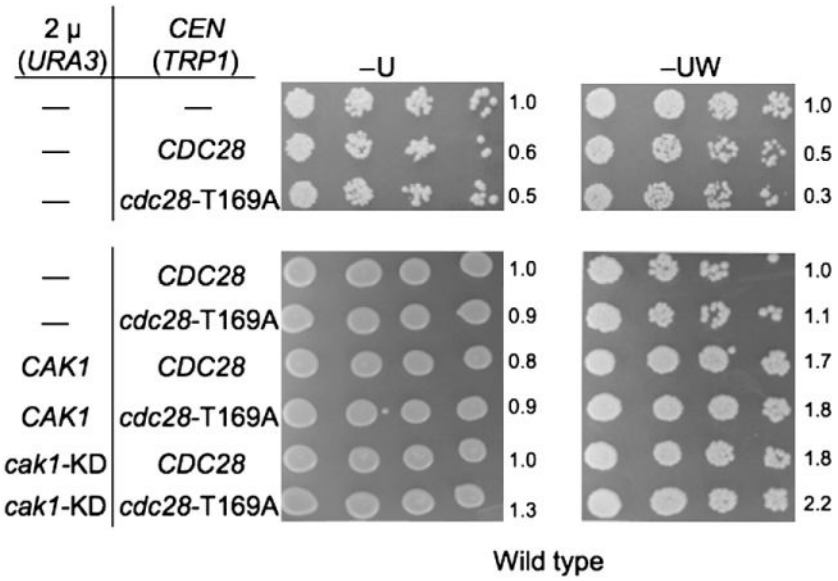

Fig. 6.

Deleterious effect of a *CEN* plasmid carrying *CDC28* or *cdc28-T169A* in wild type cells is suppressed by increasing *CAK1* or *cak1*-KD copy numbers. Wild type cells were sequentially transformed as indicated in Fig. 5B.

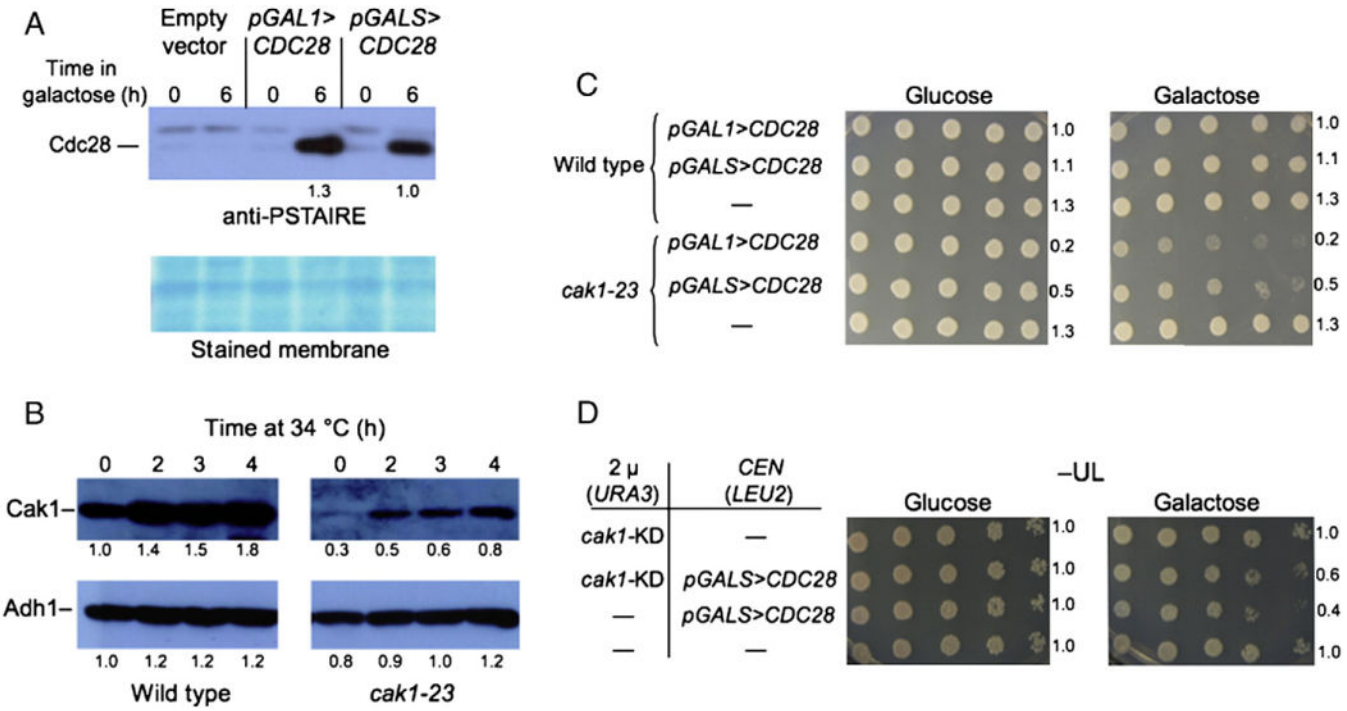

Fig. 7.

Overexpression of Cdc28 increases the temperature sensitivity of *cak1–23*. (A) Wild type cells transformed with plasmids carrying no insert, *pGAL1>CDC28* or *pGALS>CDC28* were grown overnight in raffinose and treated with galactose for the indicated times. Levels of Cdc28 protein were determined by Western blot analysis using anti-PSTAIRE antibody. (B) Lower Cak1 levels in the *cak1–23* mutant at both permissive and restrictive temperatures. Overnight cultures at room temperatures (0 h) were shifted to 36 °C and aliquots taken at the indicated times. Protein extracts were prepared and analyzed by Western blotting using monoclonal anti-Cak1 antibody. (C) Wild type and *cak1–23* harboring the indicated plasmids were grown to saturation, serially diluted (2-fold) and 5 μl aliquots spotted on either glucose or galactose-containing media. (D) *cak1–23* transformants harboring the indicated plasmids were grown to saturation. Cell suspensions were serially diluted (3-fold) and 5 μl aliquots spotted on the indicated media containing either glucose or galactose. Plates were incubated at 25 °C.

Table 1

Strains used in this study.

Name	Description	Reference
AKY2678	*cak1–23*	Espinoza et al. (1998); Ganem et al. (2006)
AKY3136	*cak1–4*	Ganem et al. (2006)
AKY2671	*cak1::TRP1* pRS316-*cak1–17*	Wagner et al. (1997)
AKY1833	*cdc28-cst2*:His3MX6 (Ala290Gly Ile291Lys Pro293Ser Tyr294Ser Ser298Pro)	Kitazono and Kron (2002)
AKY1978	*cdc28-cst3*:His3MX6 (Asn282Gly Ala290Val Ile291Cys Pro293Phe Phe295Glu Gln296STP)	Kitazono and Kron (2002)
AKY3991	*cdc28-cst8*:His3MX6 (Ala290Gly His292Leu Pro293Ala Gln296Ser)	Kitazono and Kron (2002)
AKY4294	Two-hybrid assay *MATa/MATalpha trp1–901/trp1–901 leu2–3,112/leu2–3,112 ura3–52/ura3–52 his3–200/his3–200 gal4D/gal4D gal80D/gal80D GAL2>ADE2/GAL2>ADE2 LYS2::GAL1>HIS3/LYS2::GAL1>HIS3 met2::GAL7>lacZ/met2::GAL7*N*lacZ*	James et al. (1996)

Table 2

Plasmids used in this study.

Name	Description	Reference
pAKY3983	*pGAL1*>GST-*CAK1*	Chun and Goebl (1997)
pAKB2757	*CAK1* in pRS202 (subclone)	Chun and Goebl (1997)
pAKB4308	*CAK1* in pRS424 (subclone)	Chun and Goebl (1997)
pAKB2760	*cak1*-D179N in pRS202 (*cak1*-KD, subclone)	Chun and Goebl (1997)
pAKB4308	*cak1*-D179N in pRS424 (*cak1*-KD, subclone)	Chun and Goebl (1997)
pGBDU	Bait vector two-hybrid assay (*URA3*)	James et al. (1996)
pGAD	Prey vector two-hybrid system (*LEU2*)	James et al. (1996)
pFA6a-kanMX6-pGAL1-3HA		Longtine et al. (1998)
pFA6a-kanMX6-pGAL1		Longtine et al. (1998)
pAKB3420	*pGAL1*>3HA-28CT	This study
pAKB4192	*CDC28* in pGBDU-C1	This study
pAKB4191	*CDC28* in pGAD-C1	This study
pAKB4240	*CDC28* in pRS316	This study
pAKB2257	*CAK1* in pGAD-C3	This study
pAKB4201	*CAK1* in pGBDU-C3	This study
pAKB2289	*CKS1* in pGAD-C1	This study
pAKB2303	*cdc28-cst2* in pGBDU-C1	This study
pAKB2312	*cdc28-cst8* in pGBDU-C1	This study
pAKB3958	*CDC28*-HA in *CEN TRP1*	Ross et al. (2000)
pAKB3959	*cdc28*-T169A-HA in *CEN TRP1*	Ross et al. (2000)
pSKB480	*pGAL1>CDC28*	This study
pSKB481	*pGALS>CDC28*	This study
pSKB419	*pGAL1*N*cak1*-D179N	This study
pSKB508	*pGALS>cak1*-D179N	This study
pSKB417	*pGALS>CAK1*	This study
pSKB478	*pGALS>CAK1*	This study