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A Dinoflagellate AAA Family Member Rescues a Conditional Yeast G1/S Phase Cyclin Mutant through Increased *CLB5* Accumulation

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An AAA protein from the dinoflagellate *Gonyaulax polyedra* (GpAAA) with the unusual ability to rescue the phenotype of a yeast mutant lacking G1/S phase cyclins (*cln1cln2cln3*) has been isolated and the mechanism of rescue was characterized. We find that GpAAA is not a cyclin and has no similarity to any known cell cycle regulators. Instead, GpAAA forms a novel and strongly supported clade with bacterial spoIIIAA proteins and an *Arabidopsis* gene of unknown function. Since dinoflagellates cannot be transformed, we took advantage of the powerful genetic tools available for yeast. We find that rescue of the *cln1cln2cln3* phenotype does not involve an effect on the CDK-inhibitor (CKI) Sic1p, as GpAAA does not alter the sensitivity to an inducible *SIC1*. Instead, Northern blot analyses show that GpAAA expression increases levels of *CLB5*, in agreement with the observation that GpAAA is unable to rescue the quadruple mutant *cln1cln2cln3clb5*. We propose that the increased transcription of *CLB5* may be due to a protein remodeling function of GpAAA alleviating inhibition of the transcription factor SBF. Thus, although no known equivalents to the yeast SBF have been documented in dinoflagellates, we conclude that dinoflagellates could indeed utilize GpAAA as a cell cycle regulator.

Key words: AAA protein; cell cycle regulators; cyclin; dinoflagellate; functional complementation.

Introduction

Given the wealth of knowledge on control of the cell cycle in mammalian cells and model eukaryotes such as yeast, it is surprising that almost nothing is known about control of the cell cycle in dinoflagellates. This is unfortunate, as dinoflagellates are major contributors to the phytoplankton in the oceans (Taylor and Pollingher 1987), and more importantly, rampant cell division can result in large blooms, termed red tides, that can have a serious impact on the environment and public health (Friedman and Levin 2005). Furthermore, the key mechanisms regulating the cell cycle are also of interest in order to understand how they have been modified to accommodate the characteristic permanently condensed chromosomes of dinoflagellates and the fact that the nuclear membrane remains intact through mitosis while having a cytoplasmic mitotic spindle (Moreno Diaz de la Espina et al. 2005). Unfortunately, the study of dinoflagellate biochemistry is hampered by an inability to exploit forward and reverse genetic tools, so we have instead chosen to analyze the

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function of dinoflagellate genes that affect progression of the cell cycle in yeast.

To facilitate interpretation of our analyses, a brief review of the yeast cell cycle is required. As for other eukaryotes, the cell cycle of *Saccharomyces cerevisiae* (budding yeast) is regulated by activation of the cyclin-dependent kinase Cdc28p (also termed CDK1) (Morgan 1997). This CDK is involved in regulating passage through both the G1/S phase boundary and the G2/M phase boundary. The passage between each of the boundaries requires CDK activation through binding to the regulatory cyclins that are expressed at the appropriate times to allow this passage. Once activated, CDKs phosphorylate a range of substrates whose phosphorylation allows cells to initiate either DNA replication (S phase) or mitosis (M phase), and the substrate specificity is dictated, at least in part, by the cyclin subunit bound to the CDK (Loog and Morgan 2005). Three G1 cyclins, called Cln1, Cln2 and Cln3 are involved in transitions across the G1/S-phase boundary (START) (Hadwiger et al. 1989), with the *CLN3* gene acting upstream from *CLN1* and *CLN2* (Tyers et al. 1993). Budding yeast also contains six B-type cyclins, two involved in DNA replication (*CLB5* and *CLB6*) (Toone et al. 1997), two in spindle morphogenesis (*CLB3* and *CLB4*) (Richardson et al. 1992) and two in mitosis (*CLB1* and *CLB2*) (Surana et al. 1991).

There are many factors that influence CDK activity, in keeping with the primordial role of this enzyme in ensuring an orderly progression through the cell cycle (Mendenhall and Hodge 1998). In addition to the activatory interaction with cyclins, there are also inhibitory interactions with CDK inhibitors (CKIs). One example is Sic1p, which binds and inactivates Clb/Cdc28 (Schwob et al. 1994). Sic1p is normally inactivated by Cln/Cdc28 phosphorylation allowing the cell to initiate DNA replication. Furthermore, overlaid on the regulation conferred by protein—protein interactions are post-translational modifications that affect the conformation and activity of the kinase subunit. These modifications include an activatory phosphorylation of a conserved residue in the T-loop of the CDK (Lim et al. 1996), and the inhibitory phosphorylation of conserved residues in the ATP-binding pocket whose functional significance is clear in fission yeast (Enoch and Nurse 1990) yet less so in budding yeast, despite changes through the cell cycle (Amon et al. 1992).

Interestingly, recent studies have described non-cyclin proteins able to bind and activate some CDKs. In neurons, for example, CDK5 can be activated by p35 (Lew et al. 1994; Tsai et al. 1994), although the contribution of p35 activation of CDK to regulation of the cell cycle progression is not yet clear (Dhavan and Tsai 2001). In addition, members of the Ringo/Speedy family have also been shown to bind and activate CDKs (Ferby et al. 1999; Lenormand et al. 1999). The Ringo/Speedy proteins appear to play an important role in regulation of the meiotic cycle in *Xenopus* oocytes (Ferby et al. 1999). These proteins are only marginally similar to the cyclin consensus, and the mechanism whereby they activate CDKs remains unclear.

The partial redundancy of the three yeast *CLN* genes has been exploited for the development of a yeast strain (*cln1cln2cln3*), which contains only one G1/S-phase cyclin (*CLN3*), placed under control of an inducible galactose-regulated promoter. Such a strain has been used to successfully isolate cyclin homologs from a number of different organisms by functional complementation (Koff et al. 1991; Lahue et al. 1991; Leopold and O'Farrell 1991; Lew et al. 1991; Soni et al. 1995). In this technique, libraries are screened for heterologous genes able to overcome a requirement for an endogenous *CLN* in cells grown in the presence of glucose. The isolation of non-cyclin sequences through screening has been reported only once, and the *Drosophila* Cdc28p homolog that was isolated was suggested to have a *CLN*-independent function in yeast (Leopold and O'Farrell 1991). Interestingly, yeast lacking Sic1p will grow even when none of the three *CLN* genes is expressed, indicating that this CKI is essential for imposing the requirement for G1 cyclins at START (Tyers 1996).

The molecular events occurring as yeast cells pass START involve substantial changes in the pattern of gene transcription (Cho et al. 1998; Spellman et al. 1998). These changes result from activation of two transcription factors, termed MBF and SBF, each of which recognizes different binding sites in the promoters of the regulated genes (Iyer et al. 2001). Curiously, these transcription factors are not directly substrates of Cln/Cdc28 (Wijnen et al. 2002). Instead, the causal link between transcription factor activation and CDK activation may involve phosphorylation and inactivation of a repressor, such as the Whi5 repressor of SBF (Costanzo et al. 2004).

The findings reported here are a part of an ongoing program to isolate regulators of the dinoflagellate cell cycle by functional complementation (Bertomeu and Morse 2004). In this study we have characterized a dinoflagellate AAA family

member that was able to complement a G1 cyclin mutant *cln1cln2cln3* (Bertomeu and Morse 2004). This protein, termed GpAAA, lacks any homology to known cyclins. In general, members of the AAA family of ATPases are ubiquitous proteins that exploit ATP hydrolysis for degrading or inducing conformational changes in a wide range of protein substrates (Hanson and Whiteheart 2005; Sauer et al. 2004). Our analysis of the mechanism of phenotypic rescue by GpAAA is consistent with this, as our data point to a role in alleviating repression of SBF activity thus allowing *CLB5* transcription and progression through the yeast cell cycle. We advance the intriguing possibility that a similar role might be played by GpAAA within the dinoflagellates.

Results

A *Gonyaulax* AAA Protein Rescues the Yeast cln1cln2cln3 Mutant Phenotype

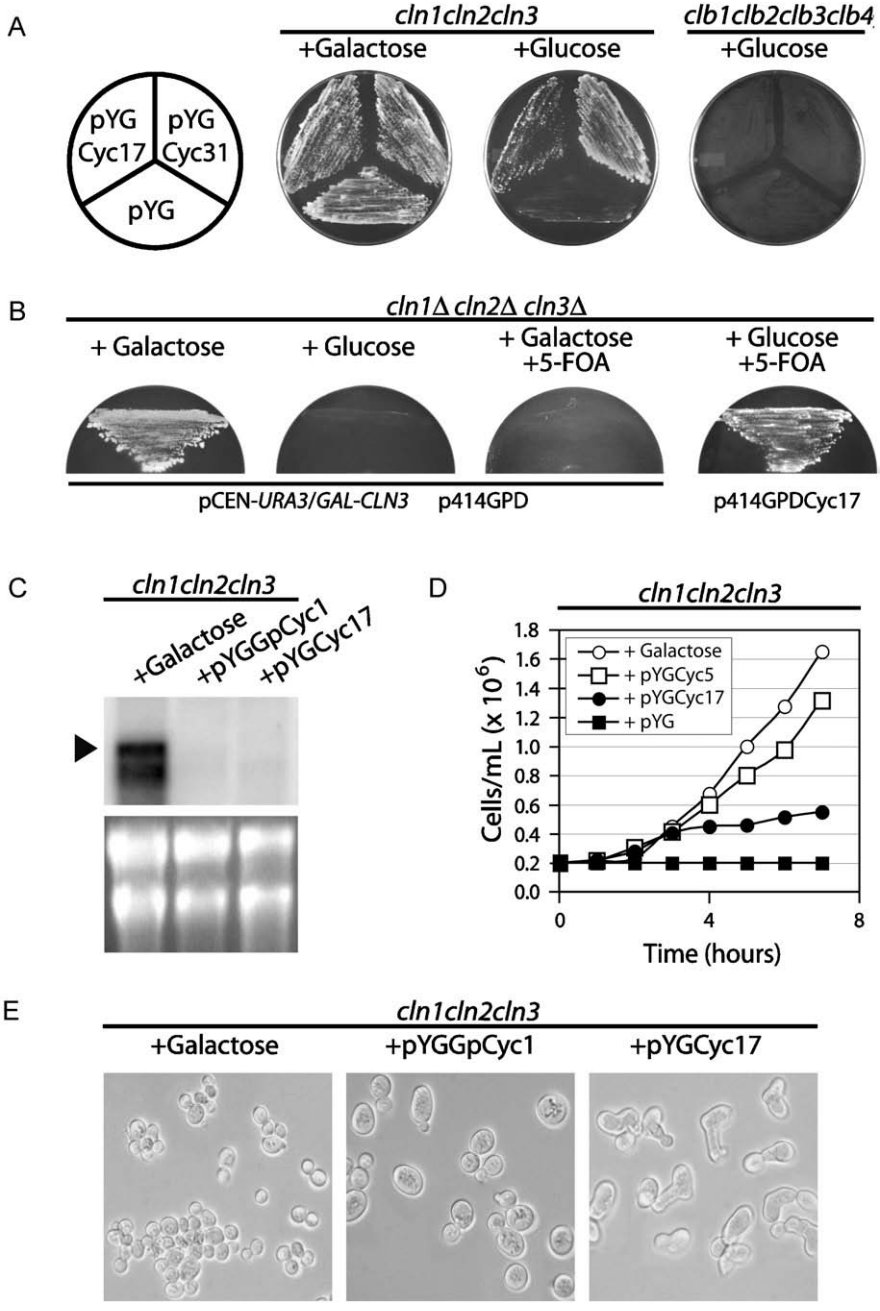
The yeast triple mutant *cln1cln2cln3* carries a mutation in all three G1/S phase cyclins, and grows in the presence of galactose due to induction of an additional *CLN3* gene under control of an inducible *GAL* promoter. This strain was transformed with a *Gonyaulax* cDNA library in a yeast expression vector, and roughly 6×10^6 clones (1.5-fold coverage of the library) were screened in the presence of glucose to inhibit normal growth. The reversion frequency of this strain on selective glucose plates is less than 10^{-6} (Xiong et al. 1991). The screen yielded 136 positive clones that were placed into one of two families of identical sequences by restriction enzyme analysis. One group, comprising 131 clones, was found to encode an authentic dinoflagellate cyclin (LpCyc1) by sequence similarity and functional analysis (Bertomeu and Morse 2004). However, the smaller second family (the remaining 5 clones) had no sequence similarity to cyclins, or indeed to any known cell cycle regulators. Sequencing revealed that the five clones isolated represented two different lengths of an otherwise identical sequence, two of 2 kb (*Cyc17* and *25*) and three of 1.5 kb (*Cyc31*, *35* and *110*). Clones of both lengths allowed proliferation of *cln1cln2cln3* (Fig. 1A) and neither were able to rescue *clb1clb2clb3clb4* (Fig. 1A) as previously found for LpCyc1 (Bertomeu and Morse 2004). The low frequency of recovery of these clones suggests that they are of low abundance, and indeed, Northern blots of poly(A)-enriched *Gonyaulax* RNA using *Cyc17* sequence as a probe did not show detectable levels of the transcript (data not shown). The longest clone recovered is ~65% GC-rich, similar to all other genes isolated from this organism.

To ensure that the rescue of *cln1cln2cln3* by *Cyc17* was not due to spurious activation of *CLN3* transcription, we transformed a *cln1Δ cln2Δ cln3Δ* bearing a *URA3* plasmid expressing *CLN3* from a *GAL* promoter, with the *Cyc17* sequence subcloned into a plasmid bearing the *TRP* marker (p414GPDCyc17). Transformants were grown for 2 days in liquid medium containing uracil but lacking tryptophan to allow the pCEN-*URA3*/*GAL*-*CLN3* to be lost, and were then plated onto medium containing 5-fluoroorotic acid (5-FOA). 5-FOA is toxic for yeast bearing the *URA3* marker, so cell growth (Fig. 1B) demonstrates that *Cyc17* acts independently of any *CLN* gene. This is in agreement with Northern analysis using *CLN3* as a probe showing no expression of *CLN3* in the *cln1cln2cln3* strain bearing pYG-Cyc17 (Fig. 1C).

Curiously, while permitting growth of the *cln1cln2cln3* mutant yeast under restrictive conditions, a complete rescue of the wild type phenotype was not obtained. For example, cells transformed with *Cyc17* have a markedly lower growth rate than do cells rescued by expression of *Cln3* or the authentic dinoflagellate cyclin *LpCyc1* (Fig. 1D). We also observe that the transformed cells have an unusual and striking morphology. Mother cells are typically more elongated than the usual rounded or slightly elliptical forms. Furthermore, while normal buds of haploid cells are also elliptical with the same general shape as the mother cell, buds in cells transformed with Cyc17 are elongated and often bulbous at their extremity (Fig. 1E). This particular phenotype has no equivalent in the yeast mutant database (http://scmd.gi.k.u-tokyo.ac.jp/datamine/) and is clearly distinct from the slightly larger but otherwise normal cells found after rescue of the mutant phenotype with the cyclin LpCyc1 (Fig. 1E).

The schematic view of the longest cDNA sequence presented here (Fig. 2A) shows the 5′ end of the two lengths of clone isolated and the position of the translational start site (ATG) immediately downstream from the end of these clones. Both of these ATG codons are in frame, and have an appropriate Kozak context (Kozak 1989). They presumably represent the start of translation since the cloning vector itself does not contain an initiation codon after the promoter. Database searches using BLASTP (Altschul et al. 1997) revealed that the N-terminal end of the

protein had no similarity to any known protein. Curiously, a block of ~75 amino acids was found repeated three times within this region (Fig. 2A—B). In contrast to the N-terminal end, a 230 residue region in the C-terminal end of the predicted protein had significant sequence identity with many hypothetical and uncharacterized ATPases from bacteria (E value $5e^{-25}$) and gene *At1g73170* from *Arabidopsis thaliana* (E value $4e^{-20}$), a higher plant. This region shares ~32% amino acid sequence identity (~52% sequence similarity) with the SpoIIIAA domain from bacteria. The function of this domain is still unknown but it is found in the first gene of operon SpoIII from many bacteria in which a defect stops sporulation at stage 3. We thus tested if either of these two domains alone could rescue *cln1cln2cln3*. A convenient Bcl1 restriction enzyme site (Fig. 2A) was exploited to clone the N and C-terminal regions separately into the



expression vector. Neither of the two regions alone was able to rescue the *cln1cln2cln3* phenotype (Fig. 2C). We also note that, since the shorter form (*Cyc31*) can also rescue, two of the three repeats in addition to the C terminal end are sufficient for activity.

The second half of the SpoIIIAA domain contains two conserved motifs (Walker A and B) that form an ATP/GTP binding site (a P-loop) (Walker et al. 1982), as well as a SRH motif (Second region of homology) (Fig. 2A—D) (Patel and Latterich 1998). These three motifs are found in members of the AAA (ATPases Associated with different cellular Activities) superfamily whose members include metalloproteases, components of the 26S proteasome, and proteins involved in membrane trafficking or organelle biogenesis. Interestingly, these different functions are reflected in primary sequence homologies and can thus be recovered using molecular phylogenetic analysis (Fröhlich 2001). Using only a small subset of sequences to represent the known different AAA family members, as well as the Cyc17, At1g73170 and various SpoIIIAA-containing bacterial sequences, we recover the same general functional classes documented previously (Fröhlich 2001) with high bootstrap support (Fig. 2E). This phylogeny clusters the Cyc17 sequence, together with the SpoIIIAA domain-containing proteins, firmly outside the previously recognized AAA groups. This suggests that both Cyc17 and the *Arabidopsis* sequence may constitute members of a new functional class of AAA proteins within the eukaryotic lineage. Taken together, our analyses indicate that the *Gonyaulax* cDNA isolated here is a *bone fide* member of the AAA superfamily and it has therefore been named GpAAA.

The Level of Sic1p Necessary to Inhibit Cell Growth is not Altered by GpAAA

We have considered three possible mechanisms by which GpAAA could allow passage through START in yeast (Fig. 3). The first mechanism is suggested by the viability of the quadruple mutant *cln1cln2cln3sic1* (Tyers 1996), as the lack of the CDK inhibitor Sic1p allows activation of the Clb5/Cdc28 complex and thus enables the mutant to overcome the normal requirement for Cln1, Cln2 and Cln3. To test if GpAAA might act to alleviate the inhibition of Cdc28/Clb5 by Sic1p (Fig. 3, mechanism 1), either by inhibiting Sic1p binding to Cdc28p or by promoting Sic1p degradation, we employed a *cln1cln2 GAL1-SIC1* strain that overexpresses this CKI when supplied with galactose. Since this strain contains a *CLN3* gene on a plasmid containing the *URA3* selectable marker, we used plasmid p414GPDGpAAA, which contains the compatible *TRP* selectable marker. GpAAA from this vector was already shown to be active (Fig. 1B). We then titrated galactose-induced expression of *SIC1* with glucose. The growth of the *cln12 GAL1-SIC1* on galactose-containing plates is inhibited at low levels of glucose, as expected (Fig. 4). More importantly, expression of GpAAA from the strong GPD promoter does not change the amount of glucose required to inhibit the growth of these cells. We conclude that GpAAA does not rescue *cln1cln2cln3* by blocking the CKI activity of Sic1p.

GpAAA Interacts only Weakly with Cdc28p in a Two-Hybrid Assay

To test for a possible interaction between Cdc28p and GpAAA, as demonstrated for other non-cyclin

Figure 1. Partial rescue of *cln1cln2cln3* mutant phenotype by a dinoflagellate cDNA. (**A**) The yeast mutant *cln1::HIS3 cln2::TRP1 cln3::URA3-GAL-CLN3* normally requires galactose for growth (left panel), although in the presence of glucose, dinoflagellate cDNAs *Cyc17* and *Cyc31* in yeast expression vector pYG can substitute for the lack of CLN3 expression (middle panel). Growth of *clb1*::*URA3 clb2*::*LEU2 clb3*::*TRP1 clb4::HIS2 GAL-CLB1-LEU2* is not rescued by either cDNA (right panel). (**B**) The yeast mutant *cln1Δ cln2Δ cln3Δ* pCEN-*URA3/GAL-CLN3* does not grow on Glucose medium or on Galactose medium supplemented with 5-FOA. Transformation with p414GPDCyc17, followed by growth for 2 days in liquid medium allowing for loss of pCEN-*URA3/GAL-CLN3*, result in yeast able to grow on Glucose supplemented with 5-FOA, demonstrating rescue in the complete absence of *CLN3*. (**C**) Northern blot using *CLN3* as a probe on 5 μg total RNA obtained from *cln1cln2cln3* grown either with galactose (left lane), or with glucose after transformation with the authentic dinoflagellate cyclin pYGLpCyc1 (middle lane) or pYGCyc17 (right lane). (**D**) The *cln1cln2cln3* mutant transformed with pYGCyc17 (closed circles) grows more slowly in glucose than does *cln1cln2cln3* cells transformed with pYGLpCyc1 (open squares), and more slowly than *cln1cln2cln3* cells grown in galactose (open circles). (**E**) *cln1cln2cln3* cells grown in glucose after transformation with pYGCyc17 (right panel) have an unusual extended bud phenotype not observed when cell express authentic cyclins (left and middle panels). All photographs are at the same scale.

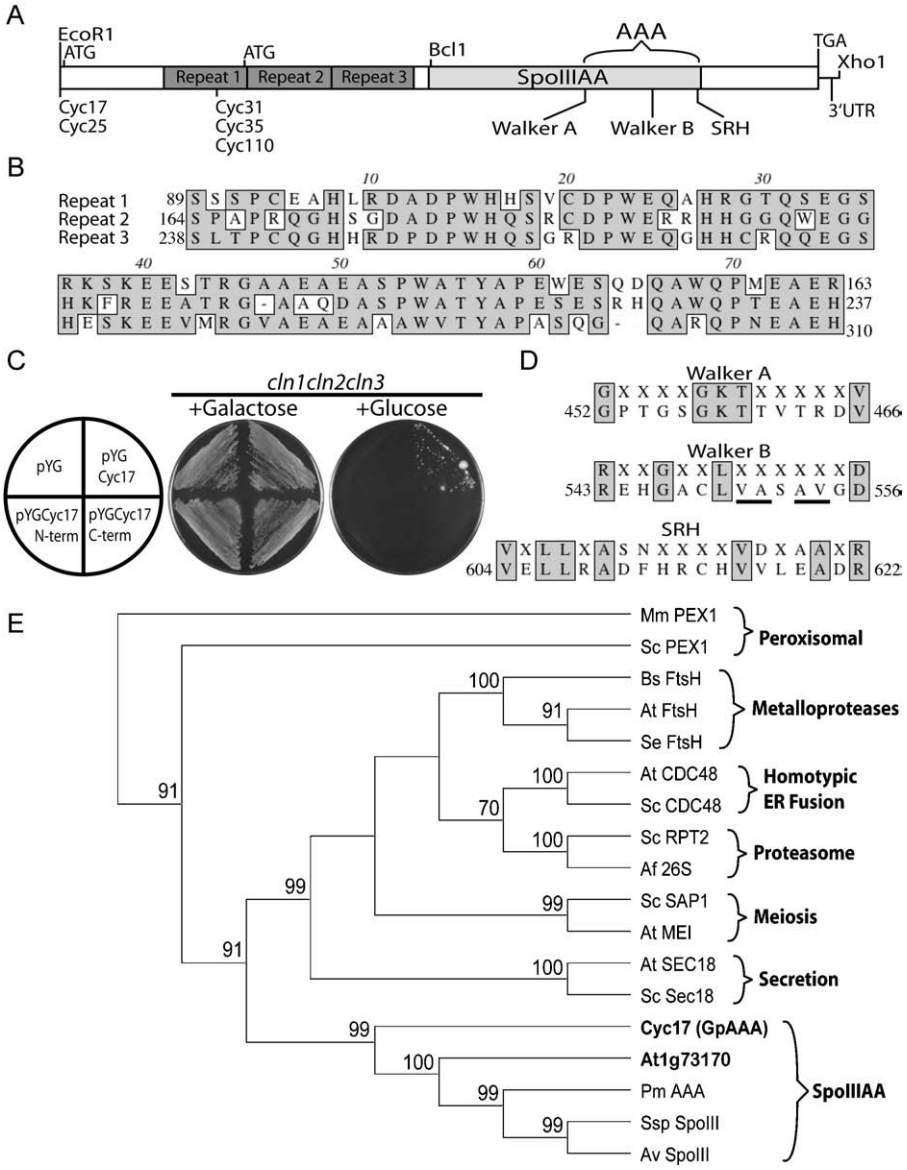


Figure 2. Cyc17 encodes an AAA protein. (**A**) A schematic view of the longest cDNA sequence showing the position of the 5′ ends of each of the clones isolated. The position of the nearest downstream start codon (ATG) for each of the two different clone lengths is shown and is predicted to produce a protein of either 656 (long clone) or 521 (short clone) amino acids. The cloning sites surrounding the insert (a 5′ EcoRI and a 3′ XhoI) as well as an internal BclI site used for subcloning are also shown. The positions of the three-repeat domain (89-310), the SpoIIIAA domain (326-556), and the signature AAA domain motifs and the predicted 3′ untranslated region (3′UTR) are illustrated. (**B**) Sequence alignment of the three N-terminal repeats domains. (**C**) Growth of *cln1cln2cln3* on glucose-containing plates is not rescued by either the N-terminal (EcoRI-BclI fragment) or the C-terminal (BclI-XhoI) domains alone. (**D**) Sequence comparisons between the Walker A, Walker B and SRH consensus motifs and the corresponding *Cyc17* sequence. Identical amino acids are boxed and characteristic hydrophobic amino acids are underlined. (**E**) Neighbor joining phylogenic reconstructions of selected AAA domains group *Cyc17* (*GpAAA*) and an *Arabidopsis* sequence of unknown function (At1g73170) in a clade distinct from previously described AAA families.

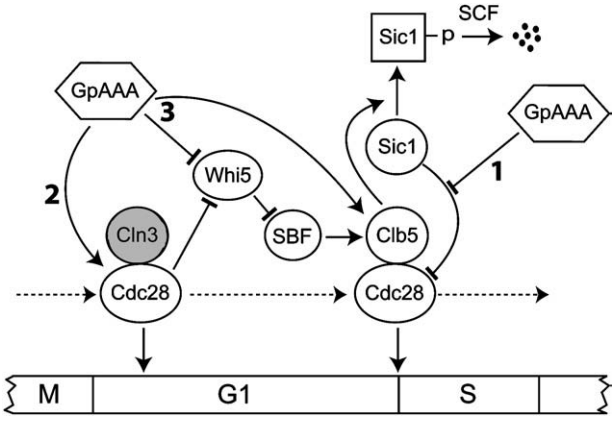


Figure 3. Schematic representation of possible mechanisms allowing GpAAA to pass the START checkpoint in the absence of CLN3. (1) GpAAA might bind and inactivate the CKI Sic1p or promote Sic1p degradation. (2) GpAAA might substitute for CLN3 in activating and targeting Cdc28p to its normal substrates. (3) GpAAA might activate *CLB5* transcription, either by direct activation of the *CLB5* transcription factor SBF or by inactivation of the SBF repressor Whi5.

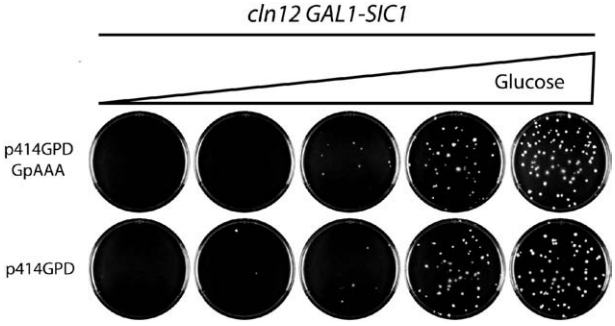


Figure 4. GpAAA does not compete with the CKI Sic1p. Expression of GpAAA does not affect the sensitivity of cell cycle progression to the level of SIC1 induced in *cln12 GAL1-SIC1* cells by varying glucose concentrations. Glucose concentrations are, from left, 0.3, 1, 3, 10 and 30 mM.

proteins such as RINGO/SPEEDY (Ferby et al. 1999; Lenormand et al. 1999), we asked if GpAAA was able to directly bind Cdc28p itself in a two hybrid assay. An interaction would be required if GpAAA were to activate Cdc28p as if it were a cyclin (Fig. 3, mechanism 2). We therefore constructed plasmids producing fusion proteins between the Activation-Domain of Gal4 and GpAAA (pADGpAAA) and between the Binding-Domain of Gal4 and Cdc28p (pBDCdc28) for use in the yeast strain PJ69-4a. This strain carries the *HIS3* and *ADE1* selectable markers under a *GAL* promoter, allowing growth if there is interaction between the two fusion proteins. We observe growth in the absence of histidine but not in the absence of adenine (Fig. 5A). Since adenine auxotrophy represents more stringent conditions than histidine auxotrophy (James et al. 1996), these results could indicate a weak interaction between Cdc28p and GpAAA. Growth on medium lacking histidine but not on medium lacking adenine has been shown in a two-hybrid assay between Clb5 and Cdc28p (Cross and Jacobson 2000). Thus mechanism 2 cannot be definitely ruled out, although it is important to stress that there is no primary sequence similarity between GpAAA and any known cyclin. Furthermore, the predicted secondary structure has nothing resembling a cyclin-fold (data not shown), in contrast to that proposed to explain the ability of p35 to activate CDK5 (Lew et al. 1994; Tsai et al. 1994).

GpAAA Rescues the cln1cln2cln3 Phenotype by Increasing CLB5 Transcript Levels

A third possibility to explain phenotypic rescue of *cln1cln2cln3* mutants by GpAAA involves activation of *CLB5* expression (Fig. 3, mechanism 3). This gene was originally isolated from a screen of *S. cerevisiae* genes able to rescue *cln1cln2cln3* (Epstein and Cross 1992). We therefore tested the ability of *GpAAA* to rescue the quintuple cyclin mutant *cln1cln2cln3clb5clb6* (Fig. 5B) and quadruple mutant *cln1cln2cln3clb5* (Fig. 5C). These strains, although rescued by overexpression of cyclin *GpCyc1*, could not be rescued by *GpAAA*. The inability of *GpAAA* to rescue the quadruple mutant indicates that *CLB6* alone is insufficient to effect rescue of the *cln1cln2cln3* phenotype. This is in agreement with the observation that *CLB5* and not *CLB6* was recovered from a screen of *S. cereviseae* genes able to overcome the *cln1cln2cln3* phenotype (Epstein and Cross 1992).

To confirm that the rescue of *cln1cln2cln3* involved *CLB5* transcription, we next tested the effect of GpAAA on expression of different G1/S activated genes. Northern blot analyses (Fig. 5D) showed a stronger expression of *CLB5* in *cln1cln2cln3* cells overexpressing *CLN3*, as expected in the presence of Galactose (de Bruin et al. 2004), as well as in *LpCyc1* and in cells bearing a *GpAAA* construct. The size of the faint signal observed within the *CLB5* probe in the *cln1cln2cln3clb5clb6* sample is indicative of

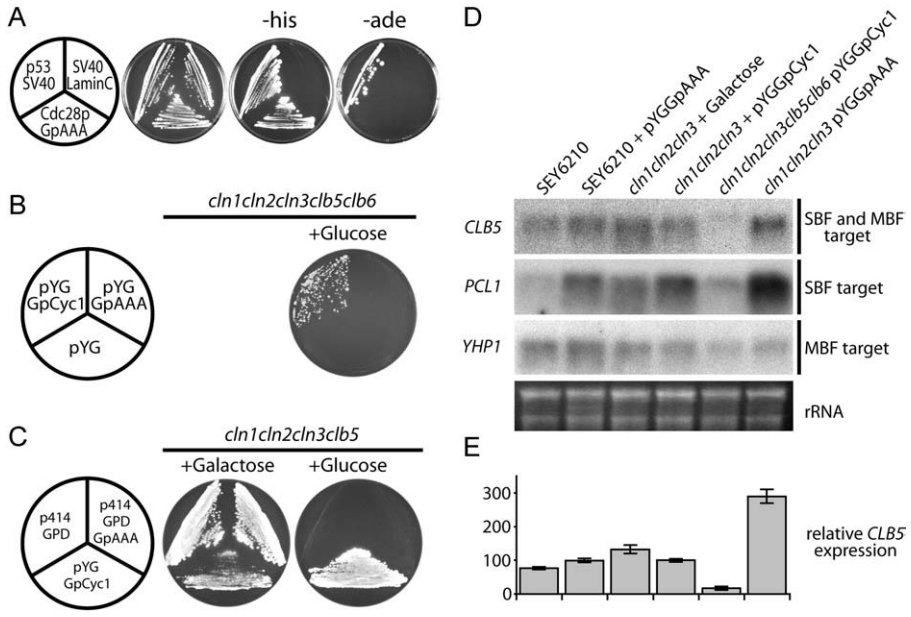


Figure 5. GpAAA activates *CLB5* transcription. (**A**) Yeast two-hybrid assay with pADGpAAA and pBDCdc28 shows a weak interaction, allowing growth on medium lacking histidine but not on medium lacking adenine. pBDp53 and pADSV40 were used as a positive control, while pBDLaminC and pADSV40 were used as a negative control. Neither pADGpAAA nor pBDCdc28p alone grow on −His plates. (**B**) pYGGpAAA does not allow growth of *cln1Δ cln2Δ cln3Δ clb5::ARG4 clb6::ADE1* pCEN-*URA3/GAL1-CLN1* in restrictive conditions. The authentic cyclin *LpCyc1* was used as a positive control and the empty vector as a negative control. (**C**) pYGGpAAA does not allow growth of *cln1Δ cln2Δ cln3Δ clb5::ARG4* pCEN-*URA3/GAL1-CLN1* in restrictive conditions. (**D**) Northern blot analysis of 4 μg of poly(A) enriched RNA of yeast strains grown in SC-medium (supplemented with galactose instead of Glucose in the third lane) probed with *CLB5*, *PCL1*, or *YHP1*. rRNA remaining in the samples was used as a loading control. (**E**) PhosphorImager quantification of accumulated CLB5 RNA levels in panel D.

hybridization with ribosomal RNA. Since *CLB5* transcription is activated by both SBF and MBF transcription factors (Bean et al. 2005), the expression of genes specifically activated by the two factors separately were also tested. The expression pattern of *PCL1*, a gene specifically transcribed at the G1/S transition and activated only by SBF (Iyer et al. 2001) mirrors that of *CLB5,* while *YHP1*, another G1/S activated gene that is exclusively an MBF target, does not (Bean et al. 2005). We conclude from this that GpAAA is likely to rescue the *cln1cln2cln3* mutant phenotype by induction of *CLB5* expression.

Discussion

We report here the isolation of a dinoflagellate AAA encoding gene GpAAA, which, when expressed in budding yeast, allows passage through the G1/S checkpoint in the absence of G1/S phase cyclins. In a functional complementation screen to rescue growth of the *cln1cln2cln3* mutant yeast, GpAAA clones were isolated five times from 6×10^6 clones, although based on their sequence, only two different clones were actually retrieved. By comparison, a dinoflagellate cyclin was recovered 131 times during the same screen (Bertomeu and Morse 2004). This shows that the GpAAA was poorly represented in the library, possibly due to weak expression in the cells. In agreement with this later idea, Northern blots using the GpAAA cDNA as a probe were unable to detect any expression of the gene. The GpAAA thus appears to be a low abundance mRNA in our dinoflagellate.

The ability of GpAAA to rescue the *cln1cln2cln3* mutant phenotype is remarkable since GpAAA does not share any sequence similarity with any known cyclins. Indeed, previous studies using libraries prepared from other organisms to screen similar mutant yeast recovered only cyclins (Koff

et al. 1991; Lahue et al. 1991; Leopold and O'Farrell 1991; Lew et al. 1991; Soni et al. 1995) with the sole exception of a *Drosophila* Cdc28p homolog that could apparently exhibit basal levels of activity without G1 cyclins (Leopold and O'Farrell 1991). The sequence of GpAAA itself yields no clue as to its function, as the only feature that can be identified is a domain found in AAA proteins. The AAA proteins have too large a spectrum of activities to provide any solid basis for predicting the function of GpAAA (Hanson and Whiteheart 2005); the only feature common to AAA family members is their ability to use ATP to induce conformational changes in specific target proteins.

To address the mechanism underlying GpAAA action in yeast, a number of different possibilities were evaluated. First, it has been previously shown that Sic1p is an inhibitor of Cdc28-Clb complexes, and that a *cln1cln2cln3sic1* yeast strain grows because Sic1p degradation is an essential function of *CLN* genes (Schneider et al. 1996; Tyers 1996). However, although members of AAA superfamily are involved in protein degradation (Hanson and Whiteheart 2005; Sauer et al. 2004), it is unlikely that GpAAA acts to degrade Sic1 because the inhibition of cell growth during titration of Sic1p expression is unaffected by the presence of GpAAA (Fig. 4).

Another possibility was that GpAAA might directly activate the CDK in a CLN-independent manner. Indeed, there are now several examples in the literature where non-cyclin proteins have been observed to activate a CDK. One of these, RINGO, activates both Cdc2 and CDK2, and plays a key role in allowing meiotic G2/M progression in *Xenopus* oocytes (Ferby et al. 1999), although the mechanism underlying this activation is still unknown. A second, SPEEDY, appear to act similarly (Lenormand et al. 1999) and may in fact be related to RINGO. A third, termed p35, has been found to activate Cdk5 in neurons (Lew et al. 1994; Tsai et al. 1994). While the physiological importance of this activation is still unclear, the mechanism may result from a similarity in the three-dimensional structure of the protein to a cyclin-fold, the domain that binds and activates CDKs, an idea supported by the similarity in the predicted alpha-helical nature of p35 and cyclins. The observation that GpAAA interacts with Cdc28p in our two-hybrid assay could potentially support CDK activation by a non-cyclin protein. However, the predicted secondary structure of GpAAA has no similarity at all to known cyclins (data not shown). Furthermore, while activation of CDK kinase activity by a non-cyclin protein is possible, it is difficult to imagine how CDK activity could then be directed toward the substrates whose phosphorylation is prerequisite for entry into S-phase entry. Lastly, and most importantly, any direct activation of Cdc28p by GpAAA would be expected to be independent of the presence of Clb5, a prediction in direct contrast to the data in Fig. 5C. These considerations lead us to conclude that a mechanism involving direct activation of Cdc28p by GpAAA is extremely unlikely.

The last possibility tested was that GpAAA might activate *CLB5* transcription in a CDK-independent manner. It was recently reported that activation of the transcription factor SBF may involve Cln3-Cdc28 dependent phosphorylation of a Whi5 repressor subunit, a mechanism conceptually similar to that of the retinoblastoma protein Rb (Costanzo et al. 2004; de Bruin et al. 2004). These new findings explain activation of SBF by Cln3-Cdc28 despite the lack of any evidence for direct phosphorylation of SBF or direct interaction between Cln3-Cdc28 and SBF (Wijnen et al. 2002). We find that GpAAA expression does indeed augment the level of *CLB5* transcript accumulation to levels above those found in all the other strains tested. The *cln1cln2cln3* rescue is clearly due to *CLB5* transcription activation as GpAAA cannot rescue *cln1cln2cln3clb5* mutant and the *CLB5* expression has already been shown to rescue a *CLN*-deficient yeast. Furthermore, the expression of *PCL1*, a specific target of SBF and not MBF, follows the same pattern as *CLB5*, while the abundance of MBF-specific genes such as *YHP1* is not significantly greater in yeast expressing GpAAA. It thus seems likely that cells expressing GpAAA have higher SBF activity without affecting the activity of MBF. Based on the role of AAA proteins in protein remodeling, we propose that this activation may be due to GpAAA-induced remodeling or degradation of Whi5.

The group of AAA proteins to which GpAAA belongs includes bacterial SpoIIIAA proteins as well as an *Arabidopsis* gene. The bacterial proteins are encoded by the first of eight genes in the spoIIIA operon, and mutants block spore formation at a stage termed engulfment (Jedrzejas and Huang 2003). Mutants blocked during this stage do not activate the specific sigma factors required to allow the continuing changes in gene expression required for completing the cell differentiation program. Although the function of SpoIIIAA is currently not known, it is an intriguing possibility that SpoIIIAA may help to modulate the changing sigma factor activities in a manner similar to the prokaryotic transcription activator

PspF, a AAA protein believed to be involved in restructuring a σ^{54}-RNA polymerase-promoter complex (Rappas et al. 2006). Thus, in bacterial systems there is clear evidence for a role of AAA proteins in regulating gene expression. Does the *Arabidopsis thaliana* At1g73170 have a similar action on transcription? Homozygous *Arabidopsis* mutants have no apparent phenotype and the *At1g73170* cDNA does not rescue the yeast *cln1cln2cln3* mutant phenotype (data not shown). However, we note that *At1g73170* has significant similarity only with the C-terminal AAA domain, and we further note that both N-terminal and C-terminal moieties of GpAAA are necessary for rescue (Fig. 2C). Thus, even if it were to act in regulating transcription, the *At1g73170* may have an alternate target not detected in our functional complementation assay. It is tempting to speculate that GpAAA may represent a new class of AAA functionalities involved in regulating transcription in both prokaryotes and eukaryotes. It is important to note in this regard that the yeast AAA protein Sap1p associates with the transcriptional repressor Sin1p (Liberzon et al. 1996), thus potentially acting to alter gene expression.

To date, the only characterized cell cycle regulator from a dinoflagellate is a cyclin most similar to mitotic cyclins (Bertomeu and Morse 2004). However, a search of the public EST databases revealed that there is at least one sequence with homology to cyclin-dependent kinases. Thus, it seems likely that dinoflagellates will use cell cycle regulators similar to those of other eukaryotes. This being the case, it is tempting to speculate that GpAAA might play a role in regulation of the dinoflagellate cell cycle that is similar that reported here in yeast. Unambiguously proving this contention will require tools not currently available, however. For example, there are no other known regulators of the dinoflagellate cell cycle, precluding tests of interactions with GpAAA. More importantly, the lack of transformation systems for dinoflagellates precludes analysis of cell behavior after introduction of a gene in sense or antisense orientations. Clearly, given these limitations, the application of yeast genetics to study of the dinoflagellate cell cycle represents a particularly valuable experimental approach.

Methods

Construction and screening of a yeast expression library: The construction of a dinoflagellate cDNA library in the yeast expression vector pYG, containing the 2 μ origin of replication and the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, has been previously described, as has the *cln1cln2cln3* mutant functional complementation protocol which yielded an authentic dinoflagellate cyclin, LpCyc1 (Bertomeu and Morse 2004). The Cyc17/GpAAA sequence described here has been deposited in GenBank with the accession number DQ515200.

Yeast strains, cultures and transformations: Mutant yeast strains used here include (i) strain SBY175 (obtained from Dr. S. Reed, Scripps Research Institute) MATa *his2 leu2 trp1 ura3 clb1*::*URA3 clb2*::*LEU2 clb3*::*TRP1 clb4*::*HIS2 GAL-CLB1-LEU2* (called *clb1clb2clb3clb4* in this study) (Richardson et al., 1992), (ii) strain BF305-15d (obtained from Dr. B. Futcher, Cold Spring Harbor Laboratories) MATa *his3 leu2 trp1 ura3 cln1*::*HIS3 cln2*::*TRP1 cln3*::*URA3-GAL-CLN3* (called *cln1cln2cln3* in this study) (Xiong et al. 1991), (iii) strain 1227-3A (obtained from Dr. F. Cross, Rockefeller University) MATα *cln1Δ cln2Δ cln3Δ* pCEN-*URA3*/*GAL-CLN3* (called *cln1Δ cln2Δ cln3Δ* in this study) (iv) strain 1851-1 (Dr. F. Cross) MATa *cln1Δ cln2Δ cln3Δ GAL1-SIC1* pCEN-*URA3*/*CLN3* (called *cln1cln2 GAL1-SIC1* in this study) (Cross and Levine 1998), (v) strain 1581-2C (Dr. F. Cross) MATα *cln1Δ cln2Δ cln3Δ clb5*::*ARG4 clb6*::*ADE1* pCEN-*URA3*/*GAL1-CLN1* (called *cln1cln2cln3clb5clb6* in this study) (Cross and Levine 1998), (vi) strain 1581-6A (Dr. F. Cross) MATa *cln1Δ cln2Δ cln3Δ clb5*::*ARG4* pCEN-*URA3*/*GAL1-CLN1* (called *cln1cln2cln3clb5* in this study); strains from the Cross laboratory are based on the A364A background (Hartwell 1967). Strain SEY6210 (from Dr. P. Belhumeur, Université de Montréal), used as wild-type for this study, MATα *his3Δ200 leu3-3,112 lys2-801 trp1Δ901 suc2Δ9 ura3-52*. Yeast were grown in synthetic complete medium (SC) lacking the appropriate auxotrophic nutrients with different amounts of glucose and/or galactose. Yeasts were transformed using a lithium acetate procedure (Gietz and Woods 2002).

Phylogenetic analysis: AAA family sequences were obtained from GenBank using a previously described tree (Fröhlich 2001) to define the major families, and aligned using CLUSTALW. Phylogenetic reconstruction was performed using Neighbor joining with absolute number of differences using MacVector (Accelrys). Bootstrap values for 10000 repetitions are reported for values of more than 70.

Plasmid construction: To produce pYGCyc17 N-term, pYGCyc17 (prepared from New England Biolabs Dam negative *E. coli* strain ER2925) was

digested with Bcl1 and Xho1 to remove the second half of *Cyc17* then recircularized by ligation with a dimerized pair of oligonucleotides (5′-GATCTGATTGGGGATCCATT-3′ and 5′-TCGAAATGGATCCCCAATCA-3′) bearing a new stop codon. To produce pYGCyc17 C-term, pYGCyc17 was digested with EcoR1 and Bcl1 to remove the first half of *Cyc17* then recircularized by ligation with a dimerized pair of oligonucleotides (5′-AATTGCCATGGGACTGGATGAGTT -3′ and 5′-GATCAACTCATCCAGTCCCATGGC -3′), bearing a new start codon and the nucleotides necessary to encode the first five amino acids of the SpoIIIAA domain that were cut with Bcl1. For pADGpAAA construction, pYGCyc17 was digested with EcoR1 and Xho1 to liberate the full length cDNA which was directionally cloned in frame in pAD also digested with EcoR1 and Xho1. For construction of pBDCdc28 *CDC28* was PCR-amplified with Pwo polymerase (5′-CATGAATTCATGAGCGGTGAATTAGCAAATTAC-3′ and 5′-TAACTGCAGTTATGATTCTTGGAAGTAGGGGTG-3′) from a plasmid bearing the complete cDNA and after digestion with EcoR1 and Pst1, ligated into EcoR1 and Pst1 digested pBDGal4. The construct p414GPDGpAAA (also called p414GPDCyc17) was prepared by directional cloning of an EcoR1 and Xho1 digestion fragment of pYGCyc17 into p414GPD (Mumberg et al. 1995), a centromeric vector containing a GPD promoter and a *TRP1* auxotrophic marker. All constructs were confirmed by sequencing.

Two-hybrid assays: The yeast strain PJ69-4a (James et al. 1996) specifically designed for two-hybrid assays was used for the Gal4-based protein-interaction experiments. It contains the markers *HIS3* and *ADE1* under a *GAL* promoter. Plasmids pBD-Gal4 and pAD-Gal4 (called pBD and pAD in this study) and pBDLaminC, pBDp53 and pADSV40 are constructs from the HybriZAP two-hybrid system (Stratagene).

Northern blots: Yeast cultures (200 ml) were grown to an O.D. 600 of 1.0, and the cells pelleted and kept at −80 °C until use. Cells were lysed in TRIzol reagent (Invitrogen) using zirconium beads and a Bead Beater and treated according to the manufacturer's recommendations. Poly(A) RNA was enriched using oligo-dT column chromatography, and either 4 μg of poly(A)-enriched RNA or 5 μg total RNA was run on formamide/formaldehyde agarose gels and transferred to positively charged nylon. The blots were hybridized against a radiolabeled probe made against a DNA fragment obtained by PCR on SEY6210 genomic DNA using the yeast Research Genetics flanking primers (YPR120C-FOR and YPR120C-REV for *CLB5*, YAL040C-FOR and YAL040C-REV for *CLN3*, YNL289W-FOR and YNL289W-REV for *PCL1*, and YDR451C-FOR and YDR451C-REV for *YHP1*). The membranes were exposed for 1 to 5 days on a PhosphorImager screen and scanned on a Typhoon 9200 (GE Healthcare).

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