

The interaction of Pcf11 and Clp1 is needed for mRNA 3'-end formation and is modulated by amino acids in the ATP-binding site

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ABSTRACT

Polyadenylation of eukaryotic mRNAs contributes to stability, transport and translation, and is catalyzed by a large complex of conserved proteins. The Pcf11 subunit of the yeast CF IA factor functions as a scaffold for the processing machinery during the termination and polyadenylation of transcripts. Its partner, Clp1, is needed for mRNA processing, but its precise molecular role has remained enigmatic. We show that Clp1 interacts with the Cleavage–Polyadenylation Factor (CPF) through its N-terminal and central domains, and thus provides cross-factor connections within the processing complex. Clp1 is known to bind ATP, consistent with the reported RNA kinase activity of human Clp1. However, substitution of conserved amino acids in the ATP-binding site did not affect cell growth, suggesting that the essential function of yeast Clp1 does not involve ATP hydrolysis. Surprisingly, non-viable mutations predicted to displace ATP did not affect ATP binding but disturbed the Clp1–Pcf11 interaction. In support of the importance of this interaction, a mutation in Pcf11 that disrupts the Clp1 contact caused defects in growth, 3'-end processing and transcription termination. These results define Clp1 as a bridge between CF IA and CPF and indicate that the Clp1–Pcf11 interaction is modulated by amino acids in the conserved ATP-binding site of Clp1.

INTRODUCTION

Poly(A) tails are added post-transcriptionally to nuclear pre-mRNA 3'-ends in eukaryotic cells in a two-step reaction involving cleavage in the 3'-untranslated region and extension of the new 3'-end by poly(A) synthesis (1,2). Polyadenylation helps the mRNA function efficiently in protein synthesis and prevents its premature degradation before it has performed this task. Besides being an essential step in mRNA maturation, it is also a point at which the cell controls gene expression (2–4). Inappropriate 3'-end processing can contribute to human diseases (5), and modulate the expression of oncogenes (6). Polyadenylation factors are also targeted by some viruses to limit expression of host cell mRNAs and favor viral protein production (7–10).

The complex that catalyzes this processing is well conserved from yeast to humans, although the individual subunits separate into somewhat different subcomplexes upon biochemical purification (1,2). In *Saccharomyces cerevisiae*, cleavage requires the 4-subunit Cleavage–polyadenylation Factor IA (CF IA), the 15-subunit Cleavage–Polyadenylation Factor (CPF) and Hrp1–Nab4, while tail synthesis requires these factors plus the Pab1 and Nab2 poly(A)-binding proteins, which restrict tail length (11,12). CPF recognizes sequences around the poly(A) site and contains the poly(A) polymerase Pap1 and the Ysh1 nuclease, which are the enzymatic activities that act at the cleavage site, while CF IA and Hrp1 help position CPF at the poly(A) site. These factors are also necessary to insure that RNA Polymerase II transcription terminates in a timely fashion and does not interfere with the function of downstream DNA elements such as promoters, centromeres and origins of replication or lead to

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the production of anti-sense RNA and possible gene silencing (13). A current challenge in the area of mRNA 3'-end processing is to improve our understanding of how the architecture of the cleavage-polyadenylation complex contributes to function and to integration of this processing machinery with other nuclear events.

In this regard, the essential Pcf11 component of CF IA is one of the better-studied subunits, and is required for both the cleavage and poly(A) addition steps. It is the only subunit of CF IA that contacts each of the other three subunits—Rna14, Rna15 and Clp1 (13–16), and it is thought to be one of the scaffold proteins of the processing complex. In support of such a role, Pcf11 also interacts with the Pta1 and Ssu72 subunits of CPF (17–19) and with the phosphorylated C-terminal domain (CTD) of the Rpb1 subunit of RNAP II (20,21), thus connecting the processing machinery to the transcription elongation complex. These interactions are mediated through a CTD interaction domain (CID) at its N-terminus, a central domain for Rna14–Rna15 interaction, and a C-terminal Clp1 interaction domain (Figure 1A). Mutations in the CID impair efficient termination of snoRNA and short mRNA transcripts, while mutation elsewhere affects the 3'-end processing and termination of long mRNAs (13–16).

Like Pcf11, the Rna14 and Rna15 subunits of CF IA are also required for 3'-end processing and termination. Rna15 recognizes the A-rich processing signal located upstream of the poly(A) site (22) and contact between its RNA Recognition Motif (RRM) and that of Hrp1 at the UA-rich signal is induced upon RNA binding (23). The tight interaction of Rna15 with Rna14 increases its affinity for RNA (24,25), and an Rna14-mediated bridge to Hrp1 helps direct Rna15 to the correct binding site (22).

In contrast to other CF IA subunits, much less is known about the precise function of yeast Clp1 other than being required for both cleavage and poly(A) addition (26). Discovered in 1997 by Minvielle-Sebastia *et al.* (27), it interacts in pull-down experiments with Cft1, Cft2, Pta1 and Pcf11 (17,19,26). Surprisingly, Clp1 is the best characterized CF IA subunit in terms of having the most complete 3D structure. It contains a large central domain that binds ATP and is flanked by two smaller N- and C-terminal domains (28). Part of the central domain surface and a hydrophobic cleft formed by the central and CTD create the Pcf11-binding site. The conserved human Clp1, and an archeal homolog, but not yeast Clp1, are 5'-OH polynucleotide kinases, and this activity in humans is important in tRNA splicing (28–32).

The goal of the work described here is to more completely define the role of Clp1 in the processing complex. In this study, we identified new interactions between Clp1 and components of the processing machinery and found that the N- and C-terminal domains of Clp1 are essential for cell viability. In addition, specific mutations in the ATP-binding site are lethal, and surprisingly, interfere with Pcf11 interaction. Mutations in Pcf11 that perturb Clp1 binding cause temperature-sensitive growth and affect cleavage, polyadenylation and transcription termination.

MATERIALS AND METHODS

Yeast strains and culture

The *S. cerevisiae* strains used in this study are as follows. Strain CM246 (MAT α Mhis3 Δ 1 leu2 Δ 1 lys2 Δ 0 ura3 Δ 0 *clp1::KanMX4* [YCpLac33-*CLP1*]) was constructed by transforming a diploid strain with a chromosomal copy of *CLP1* disrupted with *KanMX4* module (*clp1::KanMX*) with a YCpURA3-*CLP1* plasmid, and then sporulating to create the haploid strain. The *PCF11* (NA53), *pcf11-9* (NA67) and pJ69-4A strains were described previously (15,33,34). To determine the effect of *clp1* and *pcf11* mutations on yeast cell growth, the plasmid shuffle complementation assay was used (35). The pRS315 plasmids containing *clp1* mutations were analyzed in the *CLP1* plasmid shuffle strain CM246, and the pRS315 plasmids containing *pcf11* mutations were analyzed in the *PCF11* plasmid shuffle strain NA53. Growth properties were analyzed by growing the strains in liquid YPD at room temperature to an optical density at 600 nm of 1.0, spotting 5 μ l of 10-fold serial dilutions on YPD plates and incubating the plates for 3 days at 16, 24, 30, 37 or 39°C.

Saccharomyces cerevisiae *CLP1* and *PCF11* plasmids and mutants

CLP1 plasmids. For YCpLac33-*clp1*, the coding sequence of *CLP1* was amplified from genomic DNA by PCR. The PCR product was digested with NdeI and XhoI, and cloned into the YCpLac33 (*URA3*) vector flanked by the 800-bp upstream and downstream of the coding region of the *Yth1* gene. pRS315-*CLP1* and its truncations were constructed by PCR from YCpLac33-*clp1* and cloned by NheI and NcoI into pRS315 (*LEU2*). The *CLP1* sequence is flanked by a Myc epitope tag at the N-terminus and a V5 tag at the C-terminus and under the control of the *MPE1* promoter and terminator sequences (900-bp upstream and downstream of the coding region of the *MPE1* gene). Glutathione S-transferase (GST) fusion Clp1 and its truncations were constructed by insertion of PCR-amplified fragments into the EcoRI and NotI of pGEX-6 P-2. For yeast two hybrid analyses, the *CLP1* coding sequence was cloned in pGAD-C2 using EcoRI and PstI.

Pcf11 plasmids. pFL38-*pcf11* and *pcf11*-pGBDU-C1 were described previously (15,33,34,36). pRS315-*pcf11* was constructed by PCR of the coding region of *PCF11* gene from genomic DNA and cloned by NheI and NcoI into pRS315-*Clp1* digested with the same enzymes.

Clp1 and *Pcf11* site - directed mutants. Plasmids containing site-directed amino acid substitutions in Clp1 or Pcf11 were constructed using the QuikChange Mutagenesis Kit (Stratagene). All DNA fragments generated by PCR amplification in this study were verified by DNA sequencing.

Western blot analyses and immunoprecipitations

The steady-state levels of specific proteins were determined by western blot using cells grown to

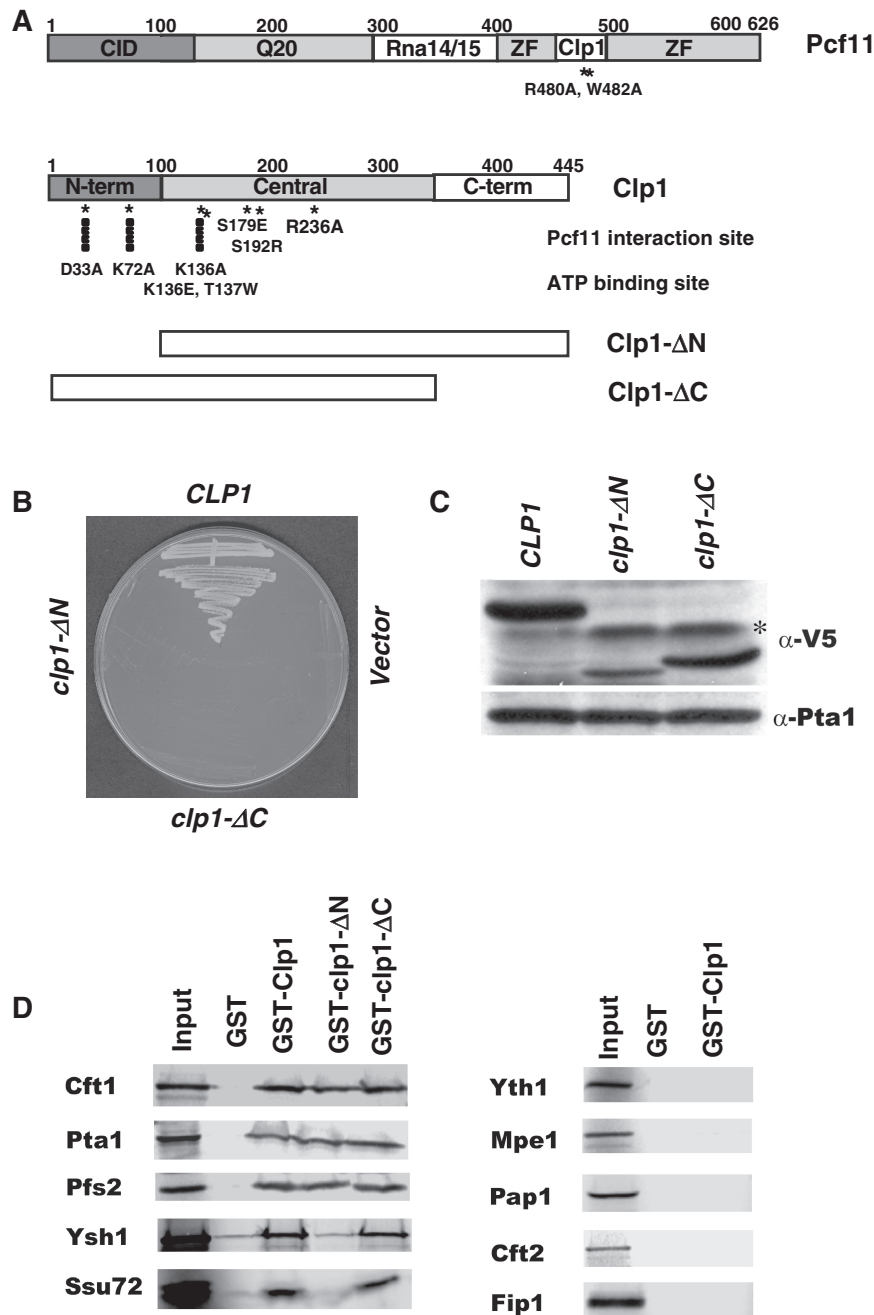


Figure 1. Functional analysis of Clp1 domains. (A) Schematic of mutations analyzed in this study. The linear representation of Pcf11 indicates the CID, the Q-rich domain (Q20), the regions which interact with Rna14, Rna15 and Clp1, the two regions containing zinc fingers (ZF), and the positions of the R480A and W482A mutations. The Clp1 representation shows the three domains determined by structural analysis (28), the N-terminal and C-terminal deletions, and the positions of mutations in the Pcf11 interaction interface and the ATP-binding site. (B) The amino and carboxyl termini of Clp1 are essential for cell viability. The CM246 [*YCpURA3-CLP1*] plasmid shuffle strain expressing epitope-tagged versions of wild-type Clp1 or the indicated deletion derivatives on a *LEU2*-marked plasmid were streaked on 5-FOA medium to counterselect the *YCpURA3-CLP1* plasmid. Plates were photographed following incubation for 4 days at 24°C. (C) *In vivo* expression of truncated forms of Clp1. Extracts prepared from strains expressing either full-length Clp1 or the indicated deletion derivatives and grown on selective medium were analyzed by western blot using monoclonal antibody against the V5 tag on Clp1 or the CPF subunit Pta1 as a loading control. Asterisk indicates non-specific band detected by the V5 antibody. (D) Clp1 interacts with specific CPF subunits. ³⁵S-labeled *in vitro*-translated subunits of CPF were incubated with GST, GST-Clp1, GST-clp1(101–445) or GST-clp1(1–341) bound to glutathione-Sepharose beads. The bound proteins and 10% of the input were separated on a 10% polyacrylamide-SDS gel and detected by autoradiography.

mid-exponential phase in liquid media and extracts prepared as described previously (37). Extracts (50 μg of protein) were resolved on an SDS–10% polyacrylamide gel, and proteins were transferred to Immobilon-P

polyvinylidene difluoride membrane (Millipore). Monoclonal antibody against Pta1 was described previously (38). Anti-V5 antibody was obtained from Invitrogen, Glutathione S-transferase (GST) antibody

from BD Biosciences-Pharmingen, GAL4-DBD (SC-510) and GAL4-TA (SC-1663) antibodies from Santa Cruz Biotechnology. Immunoprecipitations from yeast extract were performed as described by Zhelkovsky *et al.* (39).

Yeast two-hybrid analysis

pGAD-C2 vector expressing the Gal4 activation domain fused to wild-type Clp1 were transformed into the PJ69-4A host strain. The resulting strains were transformed with the pGBDU-C2 vector expressing the Gal4 DNA-binding domain fused to wild-type and mutant Pcf11. Transformants were selected on complete medium lacking uracil and leucine (CM-UL). Protein-protein interactions were scored by the ability of cells to grow on complete medium lacking uracil, leucine and histidine (CM-ULH) as described (17).

Recombinant proteins and *in vitro* protein-protein interaction assays

Radiolabeled proteins used in this study were generated *in vitro* with the TNT rabbit reticulocyte lysate system (Promega) in the presence of [³⁵S]-methionine in a total volume of 50 μ l. Expression and purification of recombinant GST-tagged proteins from the *Escherichia coli* Rosetta (DE3), as well as *in vitro* binding experiments were carried out as described previously (17,26). To produce recombinant CF IA, Rna14 and Rna15 were co-expressed from a pETDuet vector as described previously (24). Clp1 and Pcf11 were cloned into and then co-expressed from a pRSFDuet vector (Novagen). Pcf11 was cloned into the site 1 position within the vector using BamH1 and NotI restriction enzymes. Clp1 was cloned into the second position using NdeI and KpnI restriction sites. Protein expression in *E. coli* strain BL21 (DE3) pLysS was induced with 0.2 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) overnight at 16°C. Harvested cells were lysed in Tris-HCl, pH 8.0, 250 mM NaCl, 5% glycerol, 5 mM β ME, 5 mM PMSF using the Avestin C5 homogenizer and debris removed by centrifugation at 30 000g. The CF IA complex was purified using a combination of Ni-NTA, ion exchange chromatography and size exclusion chromatography. A detailed characterization of the CF IA complex will be presented elsewhere (J. Gordon, S. Shikov, J. Kuehner, M. Liriano, E. Lee, W. Stafford, C. Moore and A. Bohm, submitted for publication).

In vitro and *in vivo* RNA analyses

Preparation of yeast cell extracts, transcription of [α -³²P] UTP-labeled full-length GAL7-1 RNA or precleaved GAL7-9 RNA and processing assays have been described previously (17,38).

β -Galactosidase assay

Wild-type and mutant strains were transformed with various reporter vectors [pHZ18 Δ 2, no poly(A) site; pL101, *ADH2* poly(A) site; pL501, *GAL7* poly(A) site] (40). Cells were grown in synthetic medium lacking uracil but containing 2% galactose to an OD₆₀₀ of 0.2

and assayed by using the Gal-Screen System (Applied Biosystems).

Chromatin immunoprecipitation

Cross-linking, isolation of chromatin and sonication were performed as described previously (41). WT and mutant strains were grown to midlog (OD = 0.7–0.9) at 30°C, harvested, resuspended in prewarmed growth medium (30°C or 39°C), then incubated at either 30°C or 39°C for an additional 60 min. The PCR was performed using 2 μ l of immunoprecipitated DNA or 0.4 μ l of input DNA and the appropriate primer pairs. *PMA1* primer pairs were described previously (42). PCR products were resolved in a 1.5% agarose gel and visualized by ethidium bromide staining using an AlphaImager 2000. Rpb3 occupancy of each of the indicated regions of *PMA1* was calculated as a sample IP:input ratio and normalized to the sample IP:input ratio of an untranscribed region of chromosome V. Serial dilutions of chromatin from samples not subject to immunoprecipitation were used to demonstrate that samples are analyzed in the linear range.

ATP-binding assays

The ATP resin-binding assay was based on the protocol of Haystead, *et al.* (43). γ -Aminophenyl-adenosine triphosphate immobilized on agarose (Jena Biosciences) was equilibrated in reaction buffer (40 mM Tris, pH 8.0, 1 mM MgCl₂, 0.1% β -mercaptoethanol, 0.1% NP40, 150 mM NaCl). An amount of 500 μ l of protein (10 μ M) was incubated with 100 μ l of resin at 4°C for 1 h. The resin was washed four times with 500 μ l of reaction buffer and eluted with 500 μ l of reaction buffer + 20 mM ATP.

The ATP content of Clp1 was determined using a modification of the protocol described by Noble *et al.* (28). Equal amounts of recombinant protein were precipitated by addition of trichloroacetic acid (TCA) to 20% and the supernatant containing the released nucleotide removed and neutralized by addition of 175 mM sodium acetate (pH 8.0). Extracted nucleotides were applied to a DNA Pac PA-100 (Dionex) HPLC column equilibrated in 10 mM Tris buffer, pH 8.0. The column retention time of the nucleotide after applying a gradient of 0–1 M NaCl over 30 min was measured by monitoring the column eluent at 260 nm. Under these conditions, a control ATP sample eluted at 0.57 M NaCl.

RESULTS

Clp1 interacts with CPF subunits

The crystal structure of Clp1 with bound ATP (28) suggested that the region formed by the first 100 amino acids of Clp1 may act as a lid to cover the ATP-binding pocket of the central domain, while the C-terminal region (amino acids 341–445) contributes to Pcf11 binding (Figure 1A). To better understand the role of the Clp1 N- and C-termini, we tested the effect of deleting these domains on cell growth, using the plasmid shuffle assay. Mutants lacking the N-terminal domain (*clp1-AN*), or the

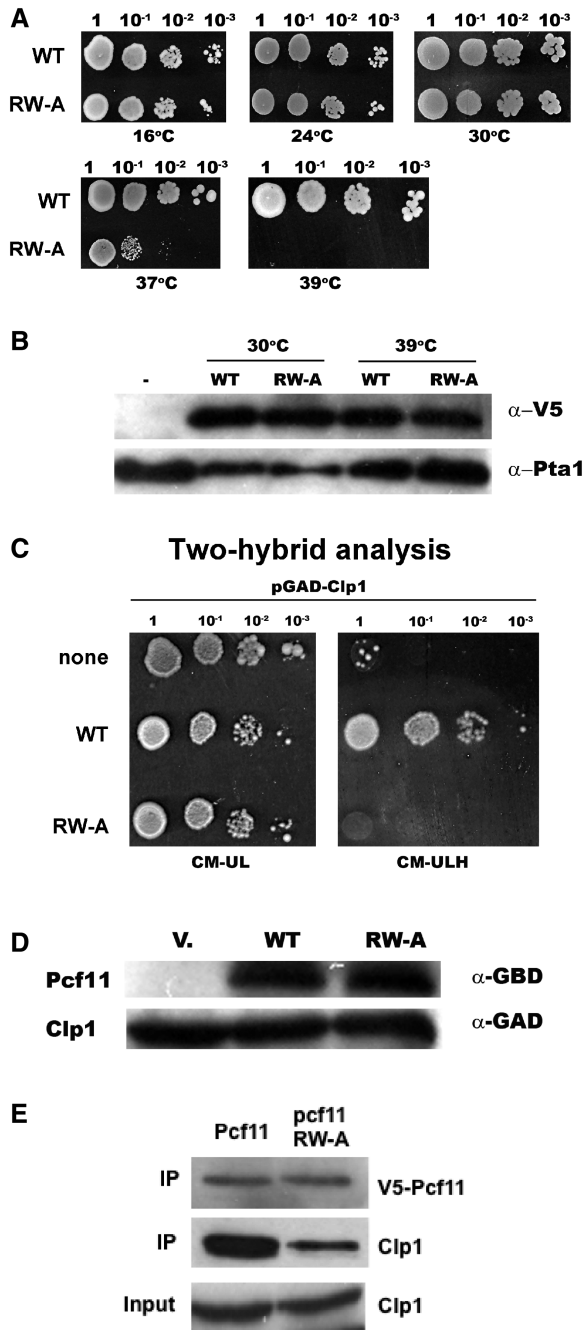


Figure 2. The *pcf11* (*RW-A*) mutant is thermosensitive for growth and defective for Clp1 interaction. (A) Relative growth rates at different temperatures. Strains were grown in liquid YPD, and 10-fold serial dilutions were spotted on YPD plates and incubated for 2–3 days at the indicated temperatures. (B) *In vivo* expression of the *pcf11* (*RW-A*) mutant. Cells containing a chromosomal deletion of the *PCF11* gene but expressing V5-tagged forms of either Pcf11 or *pcf11* (*RW-A*) or an untagged version of wild-type Pcf11 were grown at 30°C and then shifted to either 30°C or 39°C for an additional 60 min. Extracts from these cells were analyzed by western blot using a monoclonal antibody against the V5 tag or Pta1. (C) Two-hybrid analysis of the interaction between *pcf11*(*RW-A*) and Clp1. For two-hybrid analysis in yeast, plasmids carrying Pcf11, *pcf11* (*RW-A*) fused to the Gal4 DNA-binding domain (GBD), or only the GBD (none) and plasmid carrying Clp1 fused to the Gal4 activation domain (pGAD-Clp1) were cotransformed into strain PJ69-4A. Ten-fold serial dilutions of the resulting strains were spotted on medium lacking uracil and leucine (CM-UL), as indicated. Activation of *HIS3* expression was tested by

C-terminal (*clp1-ΔC*) amino acids were not viable when the wild-type *CLP1* plasmid was counter selected on 5-FOA medium (Figure 1B). Western blot analysis confirmed that the mutant Clp1 proteins were expressed, albeit at a reduced level for *clp1-ΔN* (Figure 1C). These results indicate an essential role for the N- and the C-termini in Clp1 function.

To further understand how Clp1 works in mRNA 3'-end formation, we next asked if Clp1 interacts directly with any of the CPF subunits. By using a GST-Clp1 fusion protein in pull-down assays, we detected clear interactions with CPF components Cft1, Pta1, Pfs2, Ysh1 and Ssu72, and no interaction with Yth1, Mpe1, Pap1, Cft2 and Fip1 (Figure 1D). Using the same assay, we found the N-terminal region of Clp1 is required for Ysh1 and Ssu72 interaction and the central region is required for Cft1, Pta1 and Pfs2 interactions (Figure 1D). This analysis suggests that Clp1 provides cross-factor connections to several CPF subunits.

Weakening the Pcf11–Clp1 interaction causes a thermosensitive growth phenotype

Based on the structure of the Clp1–Pcf11 complex (28), we made several mutations in Clp1 and Pcf11 that could disrupt interaction of the two proteins. Three substitutions (S179E, S192R and R236A) were selected on the Clp1 side of the interaction interface and introduced individually into the Clp1 coding sequence (Figure 1A). However, strains carrying these mutants as the only forms of Clp1 grew as well as wild-type on rich medium at 16, 23, 30, 37 and 39°C. If indeed the Clp1–Pcf11 interaction is important for function of the processing complex, contacts with Pcf11 through other amino acids that were not mutated may be sufficient to maintain the interaction.

There are two conserved residues in Pcf11 (R480 and W482) that are extensively involved in hydrogen bonding and hydrophobic interactions at the protein–protein interface (28). Both side chains project into pockets formed on the surface of Clp1. Site-directed mutagenesis was used to replace both of these residues with alanine. This mutant strain, *pcf11*(*RW-A*), grew like wild-type at 16, 24 and 30°C, but exhibited reduced growth at 37°C and no growth at 39°C (Figure 2A). Western blot analysis confirmed that the growth phenotypes were not due to instability of the mutant *pcf11* protein (Figure 2B).

To more directly examine the cause of the thermosensitive growth, we tested the ability of *pcf11*(*RW-A*)

spotting the same strains on medium lacking uracil, leucine and histidine (CM-ULH), and incubated for 2–3 days at 30°C. (D) Expression of GBD-Pcf11 and GAD-Clp1 analyzed by western blot. Extracts prepared from the different two-hybrid strains grown at 30°C were analyzed by western blot using a monoclonal antibody recognizing either the *GAL4* activation or DNA-binding domain. (E) Pulldowns of Pcf11/Clp1 complexes in yeast extract. Extract was prepared from cells expressing V5-tagged forms of either Pcf11 or *pcf11* (*RW-A*) and grown at 30°C. Immunoprecipitations from each extract were conducted using antibody against the V5 epitope, and blots of the precipitated proteins (IP) probed with Clp1 or Pcf11 antibody. The amount of Clp1 in the different extracts is shown in the panel marked 'Input'.

to interact with Clp1 by yeast two-hybrid analysis. Plasmids carrying the *GAL4* DNA-binding domain fused to *PCF11* (pGBDU-Pcf11) and plasmids carrying the *GAL4* activation domain fused to *CLP1* (pGAD-Clp1) were cotransformed into the two-hybrid tester strain PJ69-4A, and growth was examined at 30°C. All transformants grew well on media that selected for both plasmids, indicating that none of the fusion proteins were dominant-negative (Figure 2C). In addition, the GAD-Clp1 and GBDU-Pcf11 fusions could be detected by western blot analysis using antibodies specific to *GAL4* binding and activation domains (Figure 2D). Positive two-hybrid interactions activate transcription of a *HIS3* reporter gene and allow growth on medium lacking histidine (CM-ULH). pGAD-Clp1 did not promote growth in the presence of pGBDU vector alone. When pGAD-Clp1 was tested for interaction with the different pGBDU-Pcf11 constructs, we found that in contrast to the wild-type Pcf11, pcf11(RW-A) did not allow growth in the absence of histidine. These results indicate that Pcf11 amino acids R480 and W482 are needed for Clp1 interaction. The loss of a two-hybrid interaction at 30°C but viability of the *pcf11* mutant at the same temperature suggests that other interactions within the processing complex can compensate for a weakened Pcf11–Clp1 interaction at the lower temperature. It is also possible that the GBD and GAD fusions to Clp1 and Pcf11 interfere with such compensatory interactions, thus heightening an interaction defect when measured by two-hybrid analysis.

To further confirm the weakened interaction between Clp1 and the pcf11(RW-A) mutant protein, we performed pull-down assays from yeast extracts. The amount of Clp1 found in extract is not affected by the pcf11 mutation (Figure 2E). However, significantly less Clp1 was observed in complex with pcf11(RW-A) compared to that associated with wild-type Pcf11.

Disruption of the Pcf11–Clp1 interaction causes defects in mRNA 3'-end processing and transcription termination

To more directly examine the cause of the thermosensitive growth phenotype, we tested the functionality of the *pcf11(RW-A)* allele using *in vitro* and *in vivo* assays for 3'-end formation and transcription termination. Extracts prepared from cells expressing pcf11(RW-A) grown at 30°C showed very little processing activity in a coupled cleavage–polyadenylation assay when compared to extract isolated from a wild-type strain (Figure 3A, left panel). There was no accumulation of cleaved, unadenylated product, indicating that the cleavage step was defective. The mutant extract also exhibited much reduced activity when poly(A) addition was assayed on a precleaved substrate (Figure 3A, right panel). Both cleavage and poly(A) addition in the *pcf11(RW-A)* extract could be recovered by the addition of a recombinant CF IA complex containing Pcf11, Clp1, Rna14 and Rna15 (Figure 3B), indicating that the function of the CPF and Hrp1 processing factors were not affected by the mutation in Pcf11.

To test whether the *pcf11(RW-A)* mutation affected processing and transcription termination *in vivo*, we used a previously described system in which a poly(A) site is inserted into an intron upstream of the *lacZ* gene (40). Cells expressing wild-type Pcf11 or pcf11(RW-A) were transformed with reporter plasmids that had either the 3'-end of the *ADH2* or *GAL7* genes inserted into the intron, or as a control, one with no insertion, and levels of β -galactosidase activity determined. As expected, in wild-type cells, the presence of either poly(A) site blocked read-through transcription into the *lacZ* gene (Figure 4A). The *pcf11(RW-A)* strain showed an increase of 3-fold in the level of read-through transcription compared to the wild-type for the *ADH2* polyadenylation signal and an even greater increase (20 \times) for the *GAL7*

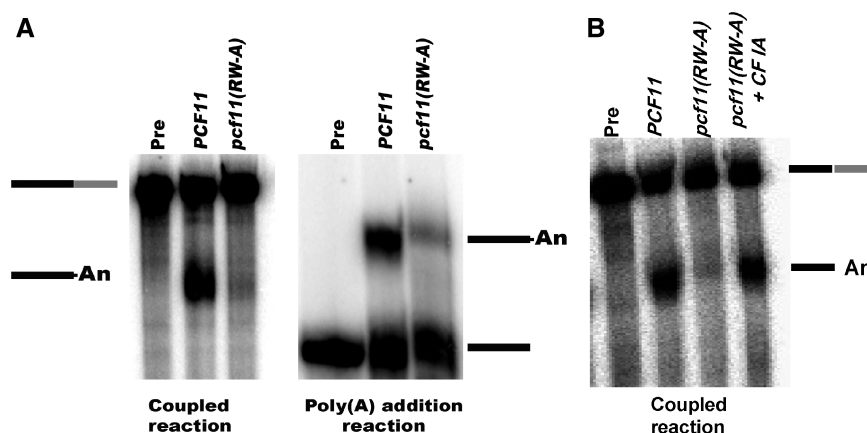


Figure 3. Mutations in the Clp1 interaction interface of Pcf11 cause defects in mRNA 3'-end formation *in vitro*. (A) *In vitro* 3'-end processing assay. For coupled cleavage-polyadenylation assays (left side), extracts prepared from the strains containing the wild-type (*PCF11*) or the *pcf11(RW-A)* mutant grown at 30°C were incubated with ATP and 32 P-labeled full-length *GAL7-1* RNA (Pre, unreacted precursor) for 20 min at 30°C. The same conditions were used for poly(A) addition assays (right side) except that the precleaved *GAL7-9* RNA was used as precursor. Products were resolved on a denaturing 5% polyacrylamide gel and visualized with a PhosphorImager. Positions of substrate and product are indicated on the left and right. (B) The processing defect of *pcf11(RW-A)* is rescued by addition of CF IA. Coupled cleavage-polyadenylation assays were performed as described in Panel A using the indicated extracts. Recombinant CF IA containing wild-type Clp1, Pcf11, Rna14 and Rna15 was added to *pcf11(RW-A)* extract before initiating the reaction (right-most lane).

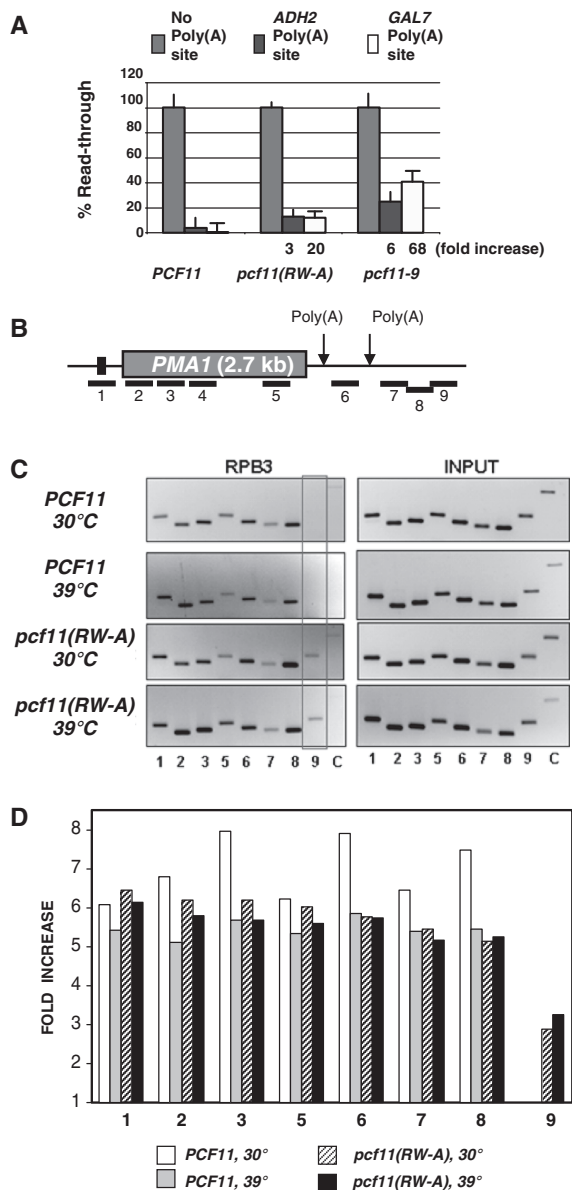


Figure 4. Mutations in the Clp1 interaction interface of Pcf11 cause defects in mRNA 3'-end formation and transcription termination *in vivo*. (A) *In vivo* 3'-end processing and termination assay. Quantitative measurements of β -galactosidase activity were performed using wild-type or mutant strains transformed with reporter vectors lacking or containing an efficient polyadenylation signal upstream of the *lacZ* gene and grown at 30°C. The amount of read-through is expressed as a percentage of that obtained from vector lacking the poly(A) site. The data is the average of 48 independent assays for each strain. The numbers under the graph indicate the fold increase in read-through transcription over the wild-type value for each poly(A) site. (B) Schematic depiction of the *PMA1* gene showing the position of its promoter (black box) and two polyadenylation sites (vertical arrows). The regions probed by ChIP are denoted 1–9. (C) Chromatin immunoprecipitation analysis of Rpb3 crosslinking to *PMA1*. Cells are grown at 30°C and then shifted to either 30°C or 39°C for an additional 60 min. before formaldehyde crosslinking. Lane C denotes a non-transcribed region of chromosome V that serves as an internal background control. All PCR primer pairs used in this analysis are identical to those described previously (41). The input signal represents DNA prior to immunoprecipitation. (D) Quantification of the data shown in panel B. Rpb3 occupancy of each of the indicated regions of *PMA1* was calculated as a sample IP:input ratio, normalized to the IP:input ratio of the untranscribed region of chromosome V (lane C in RPB3 and INPUT panels) and plotted as fold increase over background (ratio = 1.0).

poly(A) site (Figure 4A). For comparison, we used the *pcf11-9* allele, which has been previously shown to have cleavage and transcription termination defects (14,15). This mutation caused a greater read-through compared to the *pcf11(RW-A)* mutation, with a 6 \times increase for *ADH2* and 68 \times for *GAL7* (Figure 4A). It is not known why termination driven by the *GAL7* poly(A) site is more sensitive to mutations in *PCF11*.

These results suggest that the defect in 3'-end processing observed with the *pcf11(RW-A)* mutant is also causing a defect in termination. This conclusion was confirmed by determining the chromatin immunoprecipitation (ChIP) profile of RNA Polymerase II (RNAP II) across the *PMA1* gene (Figure 4B–D). In the *pcf11* mutant strain but not the wild-type, crosslinking of the RNAP II subunit Rpb3 extended into position 9, which is located downstream of the distal *PMA1* poly(A) site. Together, these analyses indicate that the Pcf11–Clp1 interaction is important for efficient 3'-end processing and termination of mRNA transcripts.

Specific alterations in the Clp1-ATP-binding site give a non-functional protein

The central domain of yeast and human Clp1 possesses a canonical P-loop (GxxxxGKT/S) which spans G130 to T137 of yeast Clp1, along with the switch regions characteristic of the SIMIBI class of ATP/GTPases (44). To probe the function of this region of Clp1, site-directed mutagenesis was employed to introduce single or double amino acid substitutions at residues in yeast Clp1 that should affect the ATP binding either at the nitrogenous base, the ribose or the phosphate (D33A, K72A, the double mutant D33A-K72A and K136A) based on the published structure (28). All of the mutants were viable in the absence of wild-type Clp1, and when tested on rich (YPD) medium at 16, 23, 30, 37 and 39°C, all of these *clp1* mutant strains exhibited growth that was indistinguishable from the corresponding wild-type strain. This finding is consistent with other studies showing that the amino acid substitutions G135R, K136A-T137A and D161A in yeast Clp1 did not affect viability (29,31). This result was surprising given the conservation of these residues and previous reports that similar active site substitutions in known ATPases abolish ATP hydrolase activity (45,46). Similar replacements also interfere with the ability of T4 polynucleotide kinase, tRNA ligases, the Grc3 RNA kinase recently implicated in termination of transcription by RNA polymerase I, and hClp1 homologs to phosphorylate RNA (30–32,47). To more rigorously test the possible importance of ATP binding, we created three additional substitutions in Clp1 (K136E, T137W and the double mutant K136E/T137W). These mutations, by substituting a positively charged amino acid for a negatively charged one (K136E) and a small amino acid for a bulky one (T137W) in the Walker A motif, were designed to be more disruptive to the ATP-binding site than alanine substitutions of the same residues. Unlike the previous mutations, none of these could support cell growth in the absence of wild-type Clp1 on 5-FOA medium (Figure 5A). Western blot confirmed that the observed

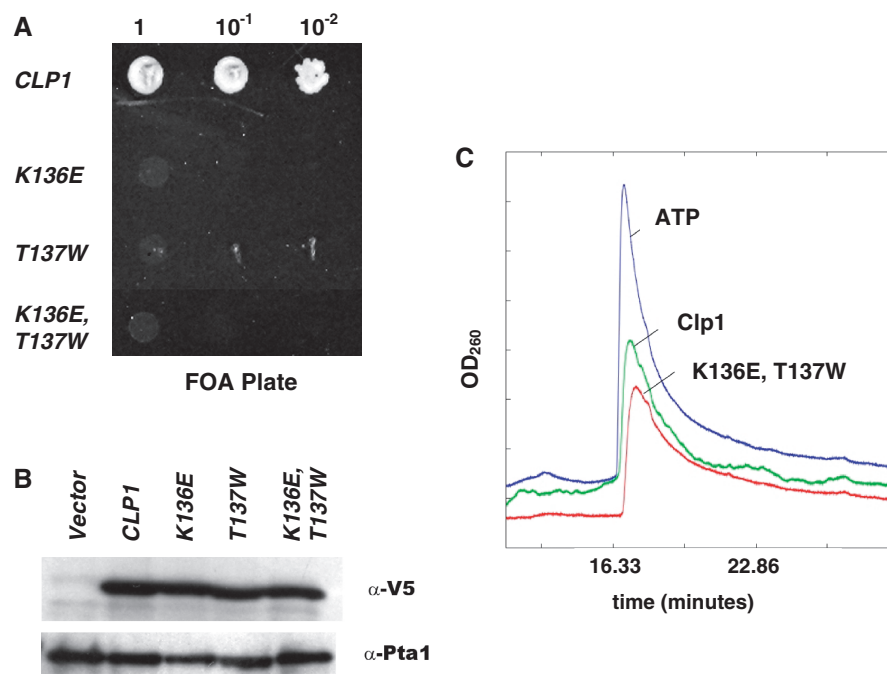


Figure 5. Analysis of mutations in the ATP-binding site of Clp1. (A) Mutations predicted to displace ATP cause inviability. The XH5 [*YCpURA3-CLP1*] plasmid shuffle strains expressing V5-tagged versions of wild-type Clp1 or the indicated mutants (K136E, T137W and K136E/T137W) were grown in selective medium, and 10-fold serial dilutions were spotted on 5-FOA plates and incubated for 4 days at 24°C. (B) *In vivo* expression of *clp1* mutants. Extracts from strains expressing either wild-type Clp1 or the indicated mutants were analyzed by western blot using monoclonal antibody against the V5 tag or the CPF subunit Pta1 as a loading control. (C) Wild-type Clp1 and the *clp1* (K136E,T137W) mutant proteins contain equivalent amounts of ATP. Equal amounts of wild-type and mutant recombinant proteins were TCA precipitated and the supernatant applied to a DNA Pac PA-100 (Dionex) HPLC column. Elution profiles were recorded at 260 nm for ATP alone and for ATP extracted from the acid-precipitated Clp1 mutant (red) and wild-type protein (green). ATP (blue) eluted at 0.57 M NaCl on a gradient of 0–1 M NaCl over 30 min. For clarity, the three traces were displaced with respect to one another on the Y-axis.

growth phenotypes were not due to instability of the mutant proteins, as the steady-state protein levels were comparable to wild-type (Figure 5B). In addition, circular dichroism analysis indicated that the K136E/T137W mutant had similar secondary structure as wild-type Clp1 (data not shown). The inviability indicates that the K136E and T137W substitutions in Clp1, which are predicted to displace ATP, interfere with one or more important functions of *S. cerevisiae* Clp1 *in vivo*.

ATP-binding assays were carried out to test whether the Clp1 double mutant (K136E, T137W) impaired ATP binding. When applied to a γ -ATP-Agarose resin column, both the wild-type and mutant proteins bound poorly, suggesting that exchange with free ATP might be very slow (data not shown). As an alternate approach, the amount of ATP bound to wild-type and mutant protein was assessed by reverse phase high-performance liquid chromatography (HPLC) analysis using recombinant protein, as described by Noble *et al.* (28). Similar amounts of ATP were extracted from the wild-type and mutant proteins (Figure 5C). This result shows that the ATP-binding capacity of the K136E, T137W protein is intact and that the inviability of the mutant is probably not due to loss of ATP binding.

To determine what might be defective about a *clp1* mutant with K136E and T137W replacements, we examined the interaction with Pcf11 by two-hybrid analysis (Figure 6A). The single mutations caused a

noticeably weaker interaction with Pcf11, and the interaction with the double mutant was significantly worse. To confirm the loss of interaction observed by two-hybrid analysis, we performed co-immunoprecipitations from yeast extracts. As expected, the *clp1* (K136E, T137W) mutant protein is defective for Pcf11 interaction in comparison to wt Clp1 (Figure 6B). These results suggest that how ATP sits in its Clp1-binding site affects specific interactions on the Clp1 surface.

DISCUSSION

The polyadenylation of mRNA 3'-ends in eukaryotes requires a surprisingly large complex, which in yeast is composed of four separate, but interacting factors, namely, CF IA, CPF, Hrp1 and Pab1 (1,2) (Figure 7A). All but two of the 21 individual subunits are essential. A challenge in the field has been to assign functions to these numerous proteins and to understand how the arrangement of proteins within the multisubunit CPF and CF IA contributes to the activity of each factor. Several subunits have enzymatic activities, such as the nuclease Ysh1, the poly(A) polymerase Pap1, and two phosphatases (Ssu72 and Glc7). Some subunits contact processing signals on the RNA precursor, while others, such as Pta1, Cft1, Pcf11 and Rna14, are thought to have scaffold functions in organizing the complex and in physically coupling processing to transcription and export (17,22,48–50). In

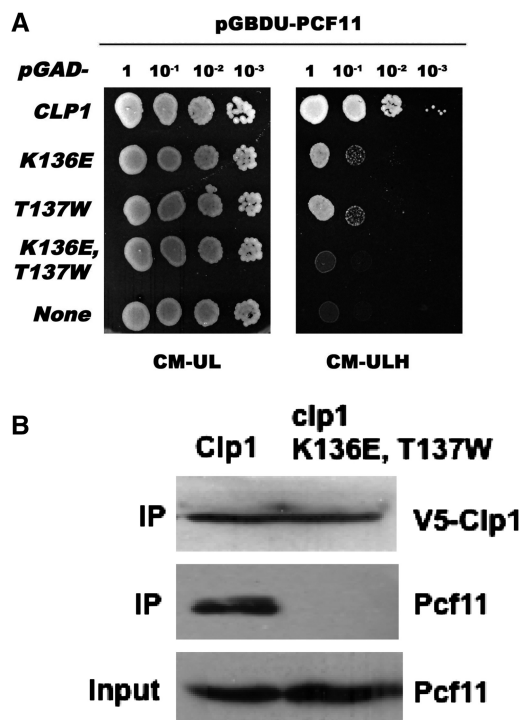


Figure 6. Clp1 proteins with K136E and T137W replacements are defective for Pcf11 interaction. (A) Two-hybrid analysis of the interaction between Pcf11 and different forms of Clp1. Two-hybrid analysis was performed as described in Figure 2C. (B) Pulldowns of Pcf11–Clp1 complexes in yeast extract. Extract was prepared from cells expressing V5-tagged forms of either Clp1 or clp1 (K136E, T137W) in addition to the untagged wt Clp1 needed for growth of the clp1(K136E, T137W) strain. Cells were grown at 30°C. Immunoprecipitations from each extract were conducted using antibody against the V5 epitope, and blots of the precipitated proteins (IP) probed with Pcf11 or Clp1 antibody. The amount of Pcf11 in the different extracts is shown in the panel marked ‘Input’.

this study, we further our understanding of the role of the Clp1 subunit of CF IA in the organization and activity of the eukaryotic polyadenylation complex and discuss the implications of our findings below.

Cross-factor contacts

Processing of mRNA 3'-ends is initiated when the factors that comprise the cleavage–polyadenylation complex recognize the constellation of signal sequences that surround the poly(A) site. Cleavage removes at least one of these signals, yet the complex must remain in stable association with the RNA so that poly(A) addition is efficient. Most of the yeast and mammalian factors do not have strong affinity for their cognate sequences, and assembly of the active complex is facilitated by cross-factor interactions. The possible proteins mediating inter-factor contacts have been most extensively investigated in the yeast system (Figure 7A). For example, the Rna14 subunit of CF IA can bridge to Hrp1 and also to CPF through association with Fip1, Pfs2 and Cft1 (22,48,51,52). Pcf11 can associate with Cft1, Cft2, Ysh1 and Pta1 (17,19) and contacts between Rna15 and Hrp1 are induced when both proteins bind RNA (23). It is important to note that

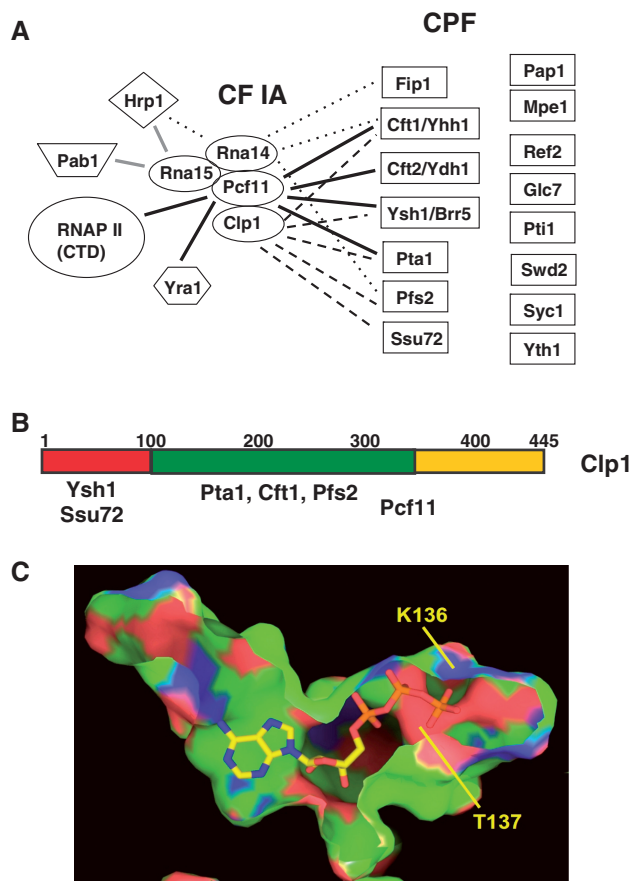


Figure 7. Regions and interactions of the yeast Clp1. (A) Summary of physical interactions known to occur between subunits of CF IA and other factors, indicated by connecting lines (Clp1 interactions, dashed line; Pcf11 interactions, solid black line; Rna14 interactions, dotted line; Rna15 interactions, gray line). These interactions include CPF subunits (19,51,52), and this study), Hrp1 (26), Pab1 (11), the CTD of the Rpb1 subunit of RNA Polymerase II (RNAP II) (20,21), and Yra1 of the mRNA export machinery (49). CPF subunits are indicated as rectangles; interactions of CF IA with CPF subunits in the right column have not been demonstrated. (B) Schematic diagram of Clp1 summarizing the regions needed for interaction with different CPF subunits. Pcf11 interacts with amino acids primarily in the central domain of Clp1 but partly in the CTD (28). (C) Model of the Clp1 ATP-binding site, based on the published structure (28). The locations of K136 and T137 are indicated. Parts of the surface composed of carbon are colored green, oxygen is red, and nitrogen is blue.

evidence for these interactions comes from *in vitro* assays using individual subunits, and none of the contacts have been confirmed to occur in an assembled processing complex.

In this study, we show that Clp1 can directly interact with the Pfs2 and Ssu72 subunits of CPF, in addition to the previously reported contacts with Cft1, Pta1 and Ysh1 (17,19), and does so through different domains (Figure 7B). However, Clp1 does not interact with Yth1, Mpe1, Pap1, Cft2 and Fip1. hClp1, a subunit of the mammalian CF II_m factor, interacts with CF I_m and CPSF (53), and the *Arabidopsis* counterpart also interacts with CPSF (54,55). CPSF contains homologs of Cft1, Cft2, Fip1, Ysh1 and Yth1. Together, these findings support the idea that one of the conserved functions of Clp1 is

to bridge different subcomplexes of the processing machinery. Interestingly, hClp1 cannot replace yeast Clp1 *in vivo* and unlike the yeast Clp1, is only needed for the cleavage step and not for poly(A) addition (31,53), suggesting important differences may exist in the protein interaction networks of these two homologs.

Consistent with the idea that the processing complex assembles on precursor RNA and then disassembles into individual factors once poly(A) addition is complete, none of the interactions of Clp1 with CPF subunits are as strong as its interaction with Pcf11, a contact conserved in the mammalian apparatus. The Pcf11-binding site is located in a groove that is partly on the surface of the central domain of Clp1 and partly at the interface of the central domain and the CTD (28). Our site-directed mutagenesis (Figure 2C) shows that this conserved interaction interface is indeed important for association of these two CF IA subunits, and that disruption of this contact interferes with CF IA function in mRNA 3'-end processing and transcription termination downstream of poly(A) sites (Figures 3 and 4). These findings are complementary to a concurrent study by Haddad *et al.* (R. Haddad, F. Maurice, N. Viphakone, F. Voisinnet-Hakil, S. Fribourg and L. Minvielle-Sébastien, *in press*), who showed that mutations in Clp1 that disrupt Pcf11 interaction are similarly defective for cleavage and poly(A) addition. Moreover, they found that depletion of Clp1 is detrimental to sn/snoRNA termination. This loss of Clp1 did not perturb the levels of Pcf11, Rna14 and Rna15, suggesting that Clp1, like the other CF IA subunits, also has an important role in termination of non-adenylated RNAP II transcripts.

Function of the ATP-binding site of yeast Clp1

Clp1 has been an intriguing component of the 3'-end processing complex because it contains a Walker A motif characteristic of the P-loop found in ATP or GTP-binding proteins, but no other motifs suggestive of function. Determination of the structure of Clp1 showed that it does indeed bind ATP (28), and the mammalian homolog was found to provide RNA kinase activity needed for one of the tRNA splicing pathways and for activating synthetic siRNAs for incorporation into silencing complexes (31,32). The alternate mechanism of tRNA exon ligation thought to use hClp1 does not occur in *S. cerevisiae*, and mutations in the ATP pocket that should destroy this activity had no effect on Clp1 function in yeast (31) and this study). Noble *et al.* (28) found that ATP hydrolysis by Clp1 was not activated by the other CF IA subunits, and proposed that the contacts of Clp1 with CPF might trigger nucleotide hydrolysis as well as exposure of the ATP-binding site to allow for ADP dissociation and ATP rebinding. Still, one would expect that the mutations in conserved P-loop residues would have growth defects if enzymatic activity was important for the essential process of mRNA polyadenylation.

In this study, we discovered that more disruptive mutations (K136E, T137W) in the P-loop of Clp1 were lethal, but did not result in loss of ATP binding (Figure 5). In an effort to explain this surprising result, we modeled the 3D

structure of the K136E, T137W mutant. The modeling confirmed that the mutated residues should interfere with the ATP conformation observed in the crystal structure. We also noticed that the ATP-binding site in wild-type Clp1 is significantly larger than necessary (Figure 7C). It is larger on both base and gamma phosphate ends of the nucleotide, and has an additional empty space extending away from the ribose. In addition, the phosphate-binding pocket appears both looser and less basic than is common. Surprisingly, the extra space around the phosphates is not filled with water molecules. Whereas yeast Clp1 appears to be a catalytically dead molecule, the mammalian homolog is an active kinase that phosphorylates the 5'-OH of RNA (31,32). Thus the extra space near the ATP-binding site may be a vestige of the RNA-binding site that is likely present in active homologs of yeast Clp1. However, with only 24% identity between the yeast and human proteins, further meaningful comparison will require a structure of hClp1. We have also not detected any interaction of Clp1 and RNA by UV crosslinking (22) or by non-denaturing gel electrophoresis (data not shown).

The ATP-binding cavity of Clp1 most closely resembles that of two characterized ATPases: the NifH Fe subunit of the *Azobabacter vinelandii* nitrogenase and the *E. coli* detoxification pump protein ArsA (28). ATP hydrolysis by these enzymes transmits conformational changes in the macromolecular complexes in which NifH and ArsA reside. Our analysis of the K136E, T137W mutant suggests that Clp1 may function in a similar way, but one that does not require catalysis. Our data show that the contact with Pcf11 was dramatically weakened (Figure 6), even though the ATP-binding site is well removed from the binding site of Pcf11 (28). This mutation does not abrogate nucleotide binding (Figure 5), suggesting a specific change in the conformation of the protein rather than gross misfolding of the central domain. Interestingly, Haddad *et al.* (29) have shown that nonlethal mutations in the P-loop domain of Clp1 will impair 3'-end processing if they are paired with a mutation in the Pcf11-binding region. This combination of mutations not only disrupts the binding of Pcf11 to Clp1, and but also its binding to the Rna14-Rna15 subcomplex, suggesting that the Clp1-Pcf11 interaction is modulated by how Clp1 binds ATP, which in turn causes a conformational change in Pcf11 that promotes its interaction with Rna14-Rna15.

One interpretation of these observations is that the ATP-binding site of Clp1 functions as a molecular switch in which the mode of ATP binding induces conformational changes necessary for processing activity. A repositioning of the ATP mimicked by the K136E, T137W mutations could occur naturally by opening of the lid formed by the N-terminal domain, which on its inner surface makes contact with the adenine base and 2' OH of the ribose (28). Ssu72 and the endonuclease Ysh1, two subunits of CPF essential for the cleavage step, interact with the N-terminal domain and could mediate such a repositioning, which in turn would loosen the interaction with Pcf11. It is not clear how these changes might help the complex move through a cycle of processing, but our

findings with the pcf11(RW-A) mutant (Figures 3 and 4) show that weakening the association of Clp1 and Pcf11 affects cleavage, poly(A) addition and termination downstream of poly(A) sites. In the lethal K136E, T137W mutant, we would predict that processing would be blocked at some point because the reorientation of the ATP is not reversible.

It is also interesting to note that Pcf11 is not an obligatory partner for Clp1. hPcf11, while binding Clp1 in the mRNA cleavage factor CF II_m, is not found with Clp1 in the human tRNA splicing endonuclease complex (32,53). Furthermore, the Clp1 interaction region on Pcf11 overlaps with that of the mRNA export factor Yra1 (49), and it has been proposed that Clp1 exchanges with Yra1 in a transition that leads to 3'-end processing and mRNP maturation when the transcription elongation complex reaches the end of a gene. While it is conceivable that the two proteins can bind Pcf11 simultaneously, the ATP binding of Clp1 could also have a role in switching Pcf11 partners. Consistent with this possibility, the Clp1–Pcf11 interaction is important in recruiting Rna15 to the RNAP II elongation complex (29).

In summary, our findings support a model in which Clp1 has a dynamic structural role in the assembly of the mRNA cleavage–polyadenylation complex on the poly(A) site and subsequent processing at that site.

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