Cdc48p is required for the cell cycle commitment point at Start via degradation of the G1-CDK inhibitor Far1p

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The budding yeast Cdc48p and its mammalian homologue p97 are involved in many important cellular activities. Because previous *cdc48* mutants have exclusive G2/M arrest, Cdc48p was thought to play an essential role only during mitosis. We found that Cdc48p is required for the execution of Start (a yeast cell cycle commitment point equivalent to the restriction point in mammalian cells) in both a normal mitotic cell cycle and cell cycle reentry after mating pheromone withdrawal through degradation of the G1–cyclin-dependent kinase inhibitor Far1p. Our work is the first to uncover novel roles of Cdc48p as a critical cell cycle regulator in G1, and to shed new light on cell cycle regulation of Far1p, which is the first cyclin-dependent kinase inhibitor shown to be a substrate of an essential proteolysis event mediated by Cdc48p.

Introduction

Cdc48p/p97 homologues (p97 is also termed VCP [valosin-containing protein]) belong to the AAA (ATPase associated with a variety of cellular activities) superfamily. Cdc48p/p97 has been shown to participate in a variety of cellular processes including: mitosis (Moir et al., 1982; Frohlich et al., 1991), spindle pole organization (Frohlich et al., 1991), homotypic membrane fusion (Latterich et al., 1995; Kondo et al., 1997), endoplasmic reticulum–associated protein degradation (Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002), transcription factor processing (Hoppe et al., 2000; Hitchcock et al., 2001; Rape et al., 2001), and ubiquitin–proteasome proteolysis pathways (Ghislain et al., 1996; Dai et al., 1998; Dai and Li, 2001).

Ubiquitin-mediated proteolysis plays critical roles in the cell cycle by regulating cyclin-dependent kinase (CDK) activities through degradation of CDK activators or inhibitors, thus promoting cell cycle transitions. After target proteins are multi-ubiquitinated, they are transported to the 26S proteasome for degradation. Recently, the crucial role of a chaperone-like Cdc48–Ufd1–Npl4 complex has been identified in the recognition and transport of polyubiquitin-tagged proteins, bringing them to the 26S proteasome for degradation (Meyer et al., 2000, 2002; Dai and Li, 2001; Ye et al., 2001). These studies suggest that Cdc48p regulates many cellular processes by this mechanism.

Besides mitosis, the G1 phase of the cell cycle is also controlled in part by ubiquitin-mediated proteolysis. One of the critical control points in G1 phase is the Start in yeast, which is equivalent to the restriction point in mammalian cells. After yeast cells have passed through Start, they not only initiate DNA replication but also form buds and duplicate their spindle pole. Activation of Cdc28 protein kinase by G1-specific cyclins is necessary for all of these Start events. However, Cdc48p has not been implicated in G1 control.

The previous conditional *cdc48* mutants are quite "leaky," as indicated by several cell doublings before eventual G2/M arrest at the restrictive temperatures (Moir et al., 1982; Frohlich et al., 1991), which has led to the idea that Cdc48p plays an essential role only during mitosis. By using a "tight" temperature-sensitive degron (td) mutant in *CDC48*, we found that Cdc48p is required for Start in G1 phase, as well as for mitosis. Furthermore, Cdc48p is essential for Start execution in both mitotic cell cycle and cell cycle reentry after mating pheromone removal; this function is achieved through degradation of the G1-CDK inhibitor Far1p.

Results and discussion

To study the functions of Cdc48p in the cell cycle, we constructed a *cdc48-td* mutant in a *GAL-UBR1* strain in which *UBR1*, the gene encoding the degron recognition factor, can be induced by galactose for efficient degradation of degron-tagged proteins at 37°C (Labib et al., 2000). The Cdc48-td protein in *cdc48-td* cells could be mostly degraded within 1 h at 37°C (unpublished data). The wild-type *CDC48* gene on a plasmid could completely rescue the *cdc48-td* mutant (unpublished data); strains complemented in this way were used as wild-type controls.

The wild-type control and *cdc48-td* strains were first synchronized in G1 phase with the mating pheromone α factor, and each culture was split into two halves: one half was left at the permissive temperature of 25°C as the control, while the other half was induced to degrade the Cdc48-td protein in *cdc48-td* cells at 37°C. Next, cells in the two cultures of each strain were released from α factor arrest into fresh medium at 25°C (Fig. 1 A) or 37°C (Fig. 1 B), respectively, and cell cycle progressions were monitored by flow cytometry and budding index counting at various time points after release. Wild-type cells at both 25°C and 37°C and *cdc48-td* cells at 25°C showed normal kinetics of bud formation and S phase entry and progression (Fig. 1). In contrast, most *cdc48-td* cells were unable to bud or enter S phase at 37°C (Fig. 1). Because bud formation and S phase entry are two independent cell cycle events that initiate simultaneously at the G1/S transition after cells have traversed Start, our data suggest that Cdc48p is required for the execution of Start.

In response to α factor in G1, the expression of Cln1p–Cln3p (the G1 cyclins) and activity of Cdc28p-Cln (the G1-
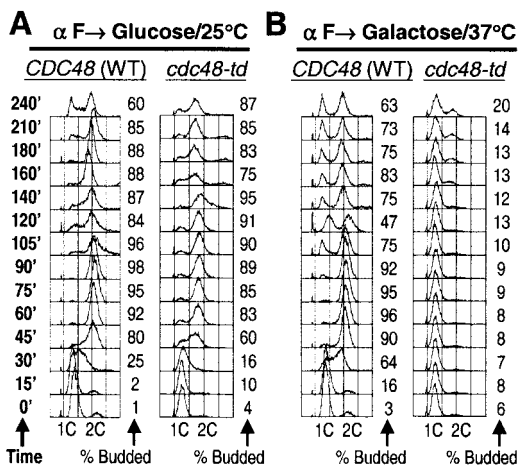
Figure 1. *cdc48-td* **cells are defective for budding and S phase entry.** Wild-type (*CDC48*) and *cdc48-td* cells were arrested in G1 with α factor at 25°C in glucose medium, and one half of culture remained in the same medium at 25°C for 2 h, while the other half was transferred into galactose medium containing α factor at 25°C to induce *UBR1* for 1 h and then shifted to 37°C for 1 h to degrade the Cdc48-td protein. Next, cells were released from α factor arrest into glucose medium at 25°C (A) or galactose medium at 37°C (B), respectively. Aliquots of cells were collected at various time points after release. Cell cycle progressions of the cells were monitored by flow cytometry and budding index counting (% Budded, percentages of budded cells in the population).
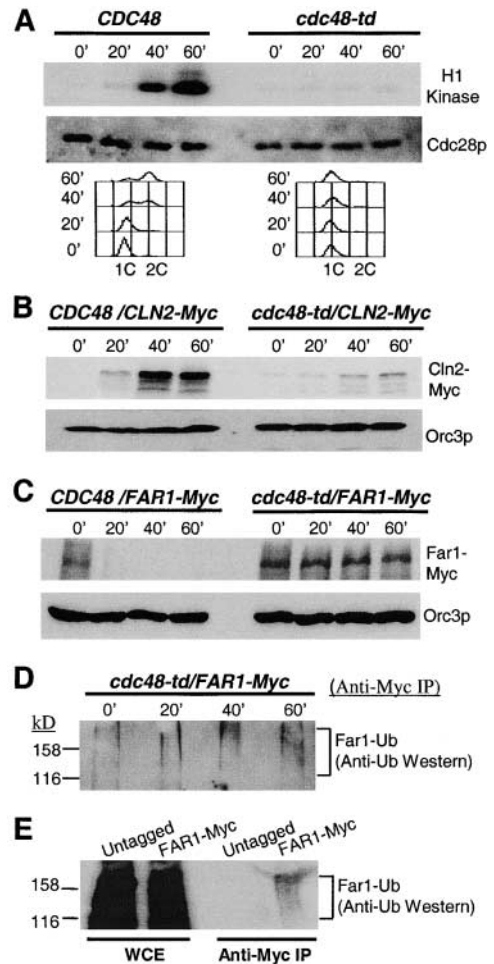
Figure 2. *cdc48-td* **cells are defective for activation of Cdc28p-Cln kinase and degradation of Far1p.** (A) Wild-type (*CDC48*) and *cdc48-td* cells were synchronized with α factor at 25°C in raffinose medium. Galactose was added to induce *UBR1* at 25°C for 1 h, and the cells were shifted to 37°C for 1 h to degrade the Cdc48-td protein. The cells were released into fresh galactose medium at 37°C for 1 h and harvested at 20-min intervals. After precipitation with p13^{Suc1} beads, the kinase activities and amounts of precipitated Cdc28p were measured by histone H1 kinase assay and immunoblotting, respectively. Cell cycle progressions were monitored by flow cytometry. (B–D) Similar experiments to those in A were performed with *CLN2-Myc* (B) or *FAR1-Myc* cells (C and D) in *CDC48* and *cdc48-td* backgrounds, and anti-Myc immunoblotting for whole cell extracts (B and C), or antiubiquitin immunoblotting for anti-Myc immunoprecipitates (D) was performed. Orc3p served as the gel loading control. (E) Extracts from *FAR1-Myc*–tagged and untagged strains were immunoprecipitated with anti-Myc, and whole cell extract and immunoprecipitates were immunoblotted with antiubiquitin.

CDK) are inhibited, and cells arrest at Start. Reentry into the cell cycle after α factor removal is achieved by recovery of the Cdc28p-Cln activities. Therefore, it is possible that the failure of *cdc48-td* mutant cells to traverse Start at 37°C after α factor withdrawal was due to the inability of the cells to recover the G1-CDK activities. To test this possibility, we blocked wild-type and *cdc48-td* cells in G1 with α factor, induced Cdc48-td proteolysis at 37°C, and then released the cells into fresh medium at 37°C. Cells were harvested at different time points after release; Cdc28p and associated proteins were precipitated by p13^{Suc1} beads (p13 is a fission

yeast CDK-binding protein that also binds the budding yeast Cdc28p) from yeast cell extracts; and the Cdc28 kinase activity was assayed using histone H1 as a substrate. In wild-type cells, Cdc28 kinase activity was absent in α factor–arrested cells (Fig. 2 A, 0 min) but gradually increased after release from the arrest, as expected. In contrast, no significant kinase activity was detected in *cdc48-td* cells after release, suggesting that recovery of the CDK activities after α factor removal requires Cdc48p.

We examined the levels of Cln2p in total yeast cell extracts in α factor arrest-and-release experiments with *CLN2-Myc* strains. In wild-type cells, Cln2p was absent in α factor–arrested cells and increased after release from the arrest, as expected (Fig. 2 B). However, in *cdc48-td* cells, Cln2p remained at a low level after release. Together, the data shown in Fig. 1 and Fig. 2 (A and B) suggest that Cdc48p is required for recovery of *CLN2* expression, for Cdc28p-Cln kinase activities, and for cell cycle reentry after α factor removal.

Because Cdc48p is known to be required for ubiquitin–proteasome proteolysis pathways, we hypothesized that inhibition of Cdc28-Cln kinase activities in *cdc48-td* resulted from failure to degrade a Cdc28p-Cln inhibitor in *cdc48-td* cells. One plausible candidate is Far1p (factor arrest), which is required for G1 arrest through inhibition of *CLN* gene expression and of Cdc28-Cln kinase activities in response to α factor (Chang and Herskowitz, 1990; McKinney et al., 1993; Peter et al., 1993; Valdivieso et al., 1993; Peter and Herskowitz, 1994). Far1p is degraded through the ubiquitin–proteasome pathway to allow cell cycle reentry after α factor withdrawal (Henchoz et al., 1997; Blondel et al., 2000).

To test if the G1 arrest was attributable to failure to degrade Far1p in *cdc48-td* cells, we examined the levels of Far1p in α factor arrest-and-release experiments with *FAR1-Myc* strains. As expected, Far1p accumulated in both wild-type and *cdc48-td* cells in the presence of α factor (Fig. 2 C, 0 min). Also as expected, the Far1p level decreased and ultimately disappeared after wild-type cells were released from α factor arrest (Fig. 2 C). However, Far1p was quite stable in *cdc48-td* cells after release from α factor arrest (Fig. 2 C). These results support our hypothesis that the inability to reenter the cell cycle resulted from failure of Far1p degradation in *cdc48-td* cells.

It has been shown that Cdc48p is responsible for bringing ubiquitinated proteins to the 26S proteosome for degradation, but it is not required for ubiquitination of protein substrates (Dai and Li, 2001; Hitchcock et al., 2001; Rape et al., 2001; Ye et al., 2001). To determine if the role of Cdc48p in Far1p degradation was also at a postubiquitination step, we used *cdc48-td* cells to check the ubiquitination status of Far1p in α factor block-and-release experiments. Far1-Myc was immunoprecipitated from yeast cell extracts with an anti-Myc antibody, and precipitated proteins were immunoblotted with an antiubiquitin antiserum. Far1-Myc was found ubiquitinated in *cdc48-td* cells at 37°C, as evident by the presence of smears (Fig. 2 D). We confirmed that the signals attributed to Far1-Ub were indeed from Far1-Ub, as smear signals could be detected by antiubiquitin immunoblotting in anti-Myc immunoprecipites from the *FAR1-Myc* tagged, but not untagged, strains (Fig. 2 E). These results suggest that Cdc48p is not required for ubiquitination of
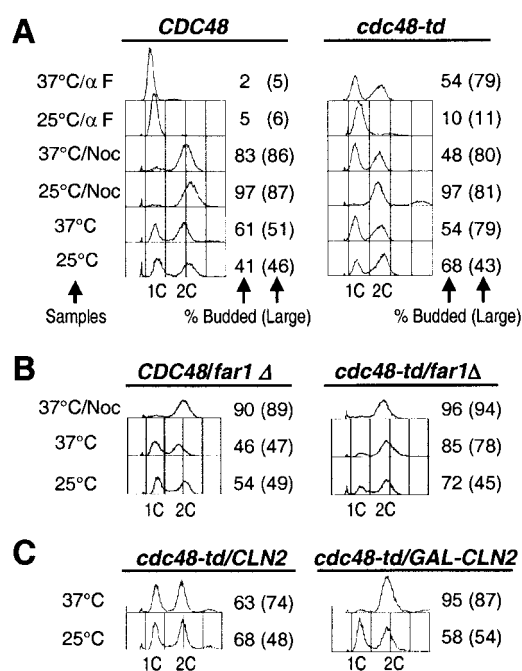
Figure 3. ***cdc48-td* cells arrest in both G1 and G2/M at 37°C, and *FAR1* deletion or *CLN2* constitutive expression can overcome the G1 arrest.** (A) Asynchronous wild-type (*CDC48*) and *cdc48-td* cells grown in glucose medium at 25°C were either transferred to galactose medium at 25°C to induce *UBR1* for 1 h and then shifted to 37°C, or kept in glucose medium at 25°C. Nocodazole or α factor was added to aliquots of cells at 25 and 37°C, and samples were harvested for flow cytometry and budding index analysis 4 h later. % Budded (Large), percentages of budded cells and of large-budded cells (in parentheses, defined as those with bud size of at least 60% of the mother) among all budded cells. (B) After *UBR1* induction, asynchronous *CDC48/far1Δ* and *cdc48-td/far1Δ* cells were shifted to 37°C in galactose medium with or without nocodazole, or kept in glucose medium at 25°C. Cells were analyzed as in A. (C) After *UBR1* induction, asynchronous *cdc48-td/CLN2* and *cdc48-td/GAL-CLN2* were shifted to 37°C in galactose medium or kept in glucose medium at 25°C. Cells were analyzed as in A.

Far1p, as in the case of other substrates whose degradation is mediated through Cdc48p.

We have established that *cdc48-td* cells are defective for Start due to failure of Far1p degradation. However, α factor was used to synchronize cells in G1 in these experiments, as in previous Far1p studies. To determine if Cdc48p was also required for degradation of Far1p at Start in the mitotic cell cycle, we examined cell cycle arrest phenotypes of *cdc48-td* cells in experiments that did not use α factor for cell presynchronization.

Asynchronous wild-type and *cdc48-td* cells were shifted to 37°C after induction of *UBR1* to degrade the Cdc48-td protein, or kept at 25°C as the control, and cell cycle distributions were examined by flow cytometry and bud index counting. Judging from the DNA contents, *cdc48-td* cells had both G1 and G2/M populations at both 25 and 37°C, as did the wild-type cells (Fig. 3 A). However, only *cdc48-td* cells at 37°C had most of their budded cells as large budded compared with a more or less equal mixture of small- and large-budded cells in wild-type cells at 25 and 37°C and *cdc48-td* cells at 25°C (Fig. 3 A). This suggests that some

cdc48-td cells arrested in G1 as unbudded cells with 1C DNA, whereas others arrested in G2/M as large-budded cells with 2C DNA at 37°C. To formally rule out the possibility that *cdc48-td* cells might have no cell cycle arrest at 37°C, nocodazole or α factor was added to yeast cell cultures just before they were shifted to 37°C (Fig. 3 A). As expected, wild-type cells at both temperatures, and *cdc48-td* cells at 25°C, were able to traverse G1 and arrest in G2/M in the nocodazole experiment, and to go through mitosis and arrest in G1 in the α factor experiment. In contrast, at 37°C in the presence of nocodazole or α factor, *cdc48-td* cells arrested in both G1 and G2/M; i.e., they could not traverse either G1 (in the nocodazole experiment) or mitosis (in the α factor experiment). Together, these results demonstrate that Cdc48p is required for cells to pass through G1, as well as mitosis, in the mitotic cell cycle.

To determine if the G1 arrest phenotype of *cdc48-td* cells not treated with α factor was also due to failure of Far1p degradation, we asked whether deletion of the *FAR1* gene could liberate *cdc48-td* cells from the G1 arrest. *CDC48* (wild-type control)/*far1Δ* and *cdc48-td*/*far1Δ* strains were used to perform experiments similar to those shown in Fig. 3 A. As expected, *CDC48*/*far1Δ* cells showed no cell cycle arrest without nocodazole, and they could be blocked in G2/M by nocodazole at 37°C (Fig. 3 B). However, unlike *cdc48-td* cells with an intact *FAR1* that have both G1 and G2/M arrest points as discussed in the previous paragraph, most *cdc48-td*/*far1Δ* cells could traverse G1 and only arrest in G2/M at 37°C with or without nocodazole (Fig. 3 B). Therefore, deletion of *FAR1* allowed *cdc48-td* cells to bypass G1 arrest even when Cdc48-td protein was degraded.

In addition to deletion of the *FAR1* gene, elimination of the Far1p-mediated inhibition of Cdc28p-Cln kinase activities can be achieved by constitutive overexpression of *CLN2* (Oehlen and Cross, 1994). By using a *cdc48-td*/*GAL-CLN2* strain in which the chromosomal copy of *CLN2* was placed under the control of a galactose inducible promoter, we tested whether *GAL-CLN2* could also allow *cdc48-td* cells to traverse Start at 37°C. Unlike *cdc48-td*/*CLN2* cells, which arrested in both G1 and G2/M at 37°C (Fig. 3, A and C), *cdc48-td*/*GAL-CLN2* cells could bypass the G1 block and only arrested in G2/M at 37°C (Fig. 3 C). Together, the results from Fig. 3 strongly suggest that the essential function of Cdc48p in Start is achieved through degradation of Far1p, leading to activation of *CLN* expression and of the Cdc28p-Cln kinase.

To determine if the action of Cdc48p in Far1p degradation was through binding to Far1p, we performed reciprocal coimmunoprecipitation (coIP) experiments between Cdc48p and Far1p. Cells were first blocked in G1 with α factor, and then released into fresh medium. Cells, harvested 7–20 min after α factor removal, were pooled for the coIP experiments. Because Far1p degradation occurs during this period, possible interactions between Cdc48p and Far1p are most likely to be detected. Both Far1-Myc and Cdc48p were detected in the anti-Myc immunoprecipitates from the *FAR1-Myc*, but not the untagged control strains (Fig. 4 A). In the reciprocal coIP, an anti-Cdc48 antiserum also precipitated both Cdc48p and Far1-Myc (Fig. 4 B). To confirm that the smear on the anti-Myc immunoblot after anti-Cdc48 immunoprecipitation (IP; Fig. 4 B, lane 3) repre-

sented ubiquitinated Far1-Myc, we performed a two-step IP experiment. First, yeast cell extracts were immunoprecipitated with anti-Cdc48. Next, the precipitated proteins were dissolved, denatured, and reprecipitated with an anti-Myc antibody. The precipitated proteins after the two-step IP were analyzed by immunoblotting using anti-Myc and anti-ubiquitin antibodies. Far1-Myc and its ubiquitinated forms were detected in the immunoprecipitates from the *FAR1-Myc*, but not the untagged control strains (Fig. 4 C). Moreover, ubiquitinated Far1p was enriched relative to un-ubiquitinated Far1p (Fig. 4 B, compare lane 3 with lane 1; and Fig. 4 C, compare lane 4 with lane 2), as has been shown for some other proteolysis substrates (Dai et al., 1998; Rape et al., 2001). These results suggest that Cdc48p participates in Far1p degradation by interacting with Far1p.
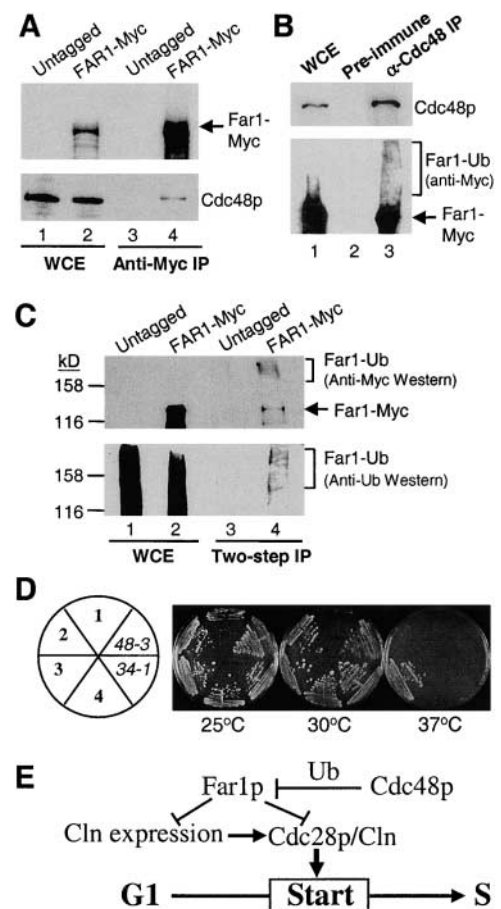
Figure 4. **Cdc48p physically interacts with Far1p and *CDC48* genetically interacts with *CDC34*.** *FAR1-Myc* (A and C) and *FAR1*-untagged (A–C) cells were arrested in G1 with α factor and released from the arrest. Cells harvested 7–20 min after release were pooled, and yeast cell extracts were immunoprecipitated with anti-Myc (A), preimmune serum (B), or anti-Cdc48 (B and C). Anti-Cdc48 immunoprecipitates in C were dissolved, denatured, and reprecipitated with anti-Myc. Immunoprecipitates were analyzed by immunoblotting with anti-Myc, anti-Cdc48, or antiubiquitin. (D) Spores of a tetratype tetrad from the *CDC48 cdc34-1/cdc48-3 CDC34* double heterozygous diploid were incubated at 25, 30, or 37°C. Spore 1, double mutant; spores 2 and 4, single mutants; and spore 3, wild type. The original single *cdc34-1* and *cdc48-3* mutants were used for comparison. (E) A model for the function of Cdc48p in G1 control. See Results and discussion for details.

Far1p degradation is mediated through the G1 ubiquitin–conjugating system composed of Cdc34p, Cdc4p, Cdc53p, and Skp1p (Henchoz et al., 1997; Blondel et al., 2000). We asked if the function of Cdc48p in Far1p degradation is through the G1 proteolysis pathway by testing if double mutants in *CDC48* and *CDC34* had synthetic lethality interactions. Haploid *cdc48-3* and *cdc34-1* single mutants in the same strain background were crossed, tetrads analysis was performed, and the segregants were incubated at 25°C. The colonies were replica plated and incubated at 25°, 30°, and 37°C to examine the growth of the spores. 19 tetrads were analyzed and 13 tetratypes, 2 parental ditypes, and 4 nonparental ditypes were found, nearly matching the expected radio. Of the 21 *cdc48–3 cdc34–1* double mutants, eight failed to form colonies and arrested growth as microcolonies, each with roughly 100 cells on tetrad dissection plates at 25°C (unpublished data). The double mutants that did form small colonies grew much more slowly than did the *cdc48-3* and *cdc34-1* single mutants at 25°C; and unlike the single mutants, the double mutants were nonviable at 30°C (Fig. 4 D). The synthetic growth defects of the *cdc48-3 cdc34-1* double mutants are consistent with Cdc48p and Cdc34p being involved in the same pathway, which is likely to be degradation of Far1p.

The G2/M arrest phenotypes of the previous *cdc48-1* mutant had led to the classification of *CDC48* as a cell division cycle gene required for mitosis (Moir et al., 1982; Frohlich et al., 1991). We have established that Cdc48p is also essential for activation of the Cdc28-Cln kinase via degradation of Far1p in G1 phase, and that degradation of Far1p is required for Start traverse in the normal mitotic cell cycle, as well as for cell cycle reentry after α factor treatment and subsequent release. Our findings demonstrate new functions of Cdc48p; i.e., to be a critical G1 regulator through degradation of Far1p, which is the first CDK inhibitor discovered as a substrate of Cdc48p-mediated proteolysis. It will be of interest and significance to examine if some other CDK inhibitors are also substrates in Cdc48p/p97-mediated proteolysis pathways in yeast and other eukaryotes.

The functions of Far1p have previously been defined only in the context of response to mating pheromone. However, the expression of *FAR1* is cell cycle regulated, with a peak at the M/G1 transition (McKinney et al., 1993), suggesting that Far1p may play a role in the normal cell cycle. Consistent with this, Far1p was found in a complex with Cdc28p-Cln in cells not exposed to pheromone, although the amount of Far1p bound to Cdc28p-Cln increased significantly when cells were treated with α factor (Peter et al., 1993; Gartner et al., 1998). Now, we conclude that efficient turnover of Far1p is necessary for cell cycle reentry after α factor withdrawal and for normal cell cycle progression, suggesting that Far1p may play a role in the mitotic cell cycle.

In the presence of α factor, phosphorylation of Far1p by the pheromone-induced MAP kinase Fus3p inhibits degradation of Far1p, leading to Far1p accumulation and G1 arrest (Peter et al., 1993). We show that if Cdc48p is absent, as in *cdc48-td* cells at 37°C, accumulation of Far1p, even in the absence of α factor, can result in cell cycle arrest in G1. Therefore, Cdc48p-mediated Far1p degradation is essential for the mitotic cell cycle. Our findings raise the intriguing possibility that Far1p may play an important role in the normal cell cycle; perhaps it ensures genomic stability, a function attributed to the Cdc28p-Clb inhibitor Sic1p (Lengronne and Schwob, 2002) and mammalian CDK inhibitors, such as p21 and p27, whose loss of function can lead to genomic instability and cancer. In such a hypothesis (Fig. 4 E), we propose that yeast cells use Far1p to inhibit the expression of *CLN* genes and Cdc28p/Cln kinase activities in early G1, until the cells are well prepared to commit to another cell cycle. Far1p is degraded through the ubiquitin–Cdc48p–proteosome proteolysis pathway, and the cells traverse Start, leading to the G1 to S transition.

Materials and methods

Construction of yeast strains

The *cdc48-td* strain was generated by inserting a HindIII–MspI *CDC48* fragment into the HindIII-ClaI sites of plasmid pPW66R (a gift from J. Diffley, Cancer Research, UK; Dohmen et al., 1994), followed by linearization within the *CDC48* sequence by SphI and integration into the *CDC48* chromosomal locus in the YKL83 strain (*ubr1Δ::GAL-UBR1*; Labib et al., 2000). To construct the *CLN2-Myc* (with 13 tandem copies of the c-Myc epitope) strain, a DNA fragment obtained by PCR with the primers OL164 and OL165, using plasmid pFA6a-13myc-kanMX6 (a gift from B. Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Longtine et al., 1998) as the template, was transformed into *cdc48-td* to generate YL559. The same strategy was used to generate the *FAR1-Myc* strain using OL173 and OL174 instead of OL164 and OL165. To generate the *GAL-CLN2* strain, the PCR product obtained with primers OL190 and OL191 and plasmid pFA6a-kanMX6-pGAL1–3HA (a gift from B. Stillman; Longtine et al., 1998) was introduced into *cdc48-td* to generate YL571. To generate the corresponding wild-type control strains, plasmid YEp213-CDC48, which was constructed by cloning a 3,946-bp MscI fragment containing *CDC48* into YEp213 by blunt end ligation, was transformed into these *cdc48-td* strains, generating YL569, YL572, and YL573, respectively. To construct the *far1Δ* strains in *CDC48* and *cdc48-td* backgrounds, PCR product obtained with primers OL172 and OL173 and plasmid pFA6a-kanMX6 (a gift from B. Stillman; Longtine et al., 1998) was introduced into YKL83 and *cdc48-td* to replace the chromosomal copy of *FAR1*, generating YL560 and YL561, respectively.

Sequences of the primers

Primers used for strain construction were as follows: OL164, 5′-agcagtgcctcatctttaatttcttttggtatgggcaatacccaagtaatacggatccccgggttaattaa-3′; OL165, 5′-ttttggtacgtttggcaaattggcattcatttatcatgaaaagaacaggaagaattcgagctcgtttaaac-3′; OL172, 5′-gtctatagatccactggaaagcttcgtgggcgtaagaaggcaatcattacggatccccgggttaattaa-3′; OL173, 5′-aaaggaaaagcaaaagcctcgaaatacgggcctcgattcccgaactagaattcgagctcgtttaaac-3′; OL174, 5′-tggtaaagcagcaaagaattcatcagaccctgaagttcccaacctccggatccccgggttaattaa-3′; OL190, 5′-ctctatagctgccaattcattcgcttaccacatcataatttgcatacagaattcgagctcgtttaaac-3′; and OL191, 5′-gatgacgagtcccatacggggtcttggttcagcactagcagccattgtgcactgagcagcgtaatctg-3′.

Cdc28 kinase assay

Soluble cell extracts were prepared by bead beating with lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM EDTA, 0.1% NP-40, 250 mM NaCl, and 1× "Complete Protease Inhibitors" added just before use; Roche). Each extract (200 µg of total proteins) was added to 15 µl p13^{Suc1}-agarose beads (Upstate Biotechnology) and incubated at 4°C for 2 h to precipitate Cdc28p. The beads were washed four times with lysis buffer. Proteins bound to half of the beads were used in immunoblotting to check the precipitation efficiency of Cdc28p. The other half of the beads was washed twice with H1 kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 1 mM DTT). Some 10 µl of kinase assay mixture (10 µCi γ-[^{32}P] ATP, 2 µg histone H1, and 100 µM ATP in 1× kinase buffer) was added to the beads, and the mixture was incubated at 25°C for 10 min. The reaction was stopped by adding 10 µl of 2× Laemmli's buffer followed by boiling. Samples were resolved by 10% SDS-PAGE gel and subjected to autoradiography.

Two-step IP

Immunoprecipitates of the first (anti-Cdc48; a gift from K. Frohlich, Physiologisch-chemisches Institut, Tubingen, Germany) IP were boiled in 50 µl

of lysis/IP buffer (Zhang et al., 2002) containing 1% SDS for 10 min. Next, solublized proteins were diluted with 450 µl of lysis/IP buffer and subjected to the second (anti-Myc) IP.

Cell synchronization, flow cytometry, immunoprecipitation, and immunoblotting

These experiments were performed as described previously (Liang and Stillman, 1997; Zhang et al., 2002).

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