Fission yeast WD-repeat protein Pop1 regulates genome ploidy through ubiquitin–proteasome-mediated degradation of the CDK inhibitor Rum1 and the S-phase initiator Cdc18

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In fission yeast, maintenance of genome ploidy is controlled by at least two mechanisms. One operates through the Cdc2/Cdc13 kinase, which also involves the CDK inhibitor Rum1, and the other through the S-phase regulator Cdc18. By screening for sterile mutants that show increased ploidy, we have identified a new gene, pop1+, in mutants that become polyploid. The pop1 mutation shows a synthetic lethal interaction with the temperature-sensitive cdc2 or cdc13 mutation. In a pop1 mutant Rum1 and Cdc18 proteins become accumulated to high levels. The high ploidy phenotype in the pop1 mutant is dependent on the presence of the rum1+ gene, whereas the accumulation of Cdc18 is independent of Rum1. The predicted sequence of the Pop1 protein indicates that it belongs to a WD-repeat family with highest homology to budding yeast Cdc4, which participates in the ubiquitin-dependent pathway. Consistent with this notion, in a mutant of the 26S proteasome, higher molecular weight forms of Rum1 and Cdc18 are accumulated corresponding to polyubiquitination of these proteins. In the pop1 mutant, however, no ubiquitinated forms of these proteins are detected. Finally we show that Pop1 binds Cdc18 in vivo. We propose that Pop1 functions as a recognition factor for Rum1 and Cdc18, which are subsequently ubiquitinated and targeted to the 26S proteasome for degradation.

[Key Words: Cell cycle; CDK inhibitor; initiation of S phase; polyploid; proteasome; fission yeast; ubiquitin]

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Maintenance of genome ploidy is one of the fundamental features for all organisms. Cells usually maintain a fixed ploidy when they divide and when they arrest at a specific stage of the cell cycle during differentiation. Ploidy is maintained by a faithful ordering between replication (S phase) and segregation (M phase) of the chromosomal DNA. S phase always precedes M phase in a single cell cycle and S phase follows M phase of the previous cycle. Coordination between S and M phase is crucial for a normal mitotic cell cycle [for review, see Nurse 1994]. Disruption of the ordering between S and M phase is deleterious for the somatic cell cycle, although variation in ploidy is observed during development [e.g., Orr-Weaver 1994].

There are several examples of circumstances that lead to polyploidization or over-replication either by chemical treatments or by mutations. For instance, in animal culture cells, the drug K-252a is shown to cause reactivation without intervening mitoses [Usui et al. 1991]. In fly, mutations either in Dmc2c or in escargot lead to polyploid cells in imaginal discs and the central nervous system [Hayashi 1996]. In Chinese hamster cell lines, the ts41 mutation results in successive S phases [Handeli and Weintraub 1992].

Recently it has become clear that protein turnover is a key mechanism for the regulation of protein levels in the cell. In particular, protein ubiquitination followed by the 26S proteasome-dependent proteolysis has crucial roles in a wide variety of cellular processes [for review, see Deshaies 1995; Hochstrasser 1995]. Ubiquitin, a highly conserved 76-residue protein, is joined covalently to the acceptor proteins via a multistep process. The reactions require a series of specific enzymes, including E1 [ubiquitin-activating enzymes], E2 [ubiquitin-conjugating enzymes], and sometimes E3 [ubiquitin–ligases]. The 26S proteasome is composed of a core protease known as the 20S proteasome and a pair of regulatory complexes, often referred to as PA700. It is generally believed that the 26S proteasome recognizes selectively polyubiquitinated proteins in an ATP-dependent manner, although there is
one case where the substrate (ornithine decarboxylase) is not ubiquitinated. Ubiquitin-dependent protein degradation has emerged as a primary mechanism that regulates crucial aspects of the cell cycle. Cyclin B is a classical substrate for the proteasome, which is abruptly degraded in anaphase. Other cyclins such as those of budding yeast and human cyclin E are also shown to be degraded by ubiquitin-dependent proteolysis (Deshaias et al. 1995; Clurman et al. 1996; Won and Reed 1996). In addition, other important regulators for sister chromatid segregation in anaphase are degraded through the same proteasome pathway (Funabiki et al. 1996; Yamamoto et al. 1996).

Fission yeast usually proliferates as haploid cells with ploidy being maintained faithfully. Several conditions are known to cause diploidization or polyploidization, which can be classified into the following three types. The first type represents bypass of M phase, which takes place in conditions leading to inactivation of Cdc2/Cdc13 (B-type cyclin), an archetype cyclin-dependent protein kinase (CDK). This includes complete disruption of the cdc13+ gene, heat shock treatment of temperature-sensitive cdc2 or cdc13 mutants, or ectopic overexpression of rum1+, which encodes a CDK inhibitor (Broek et al. 1991; Hayles et al. 1994; Moreno and Nurse 1994; Correa-Bordes and Nurse 1995). The second type comprises successive S phase in the absence of G1, G2, and M phases, which is driven by ectopic overexpression of cdc18+ encoding a critical regulator for the initiation of S phase (Kelly et al. 1993; Nishitani and Nurse 1995). The third type displays incomplete M phase, which is caused by the cut1 mutation defective in chromosome disjunction (Creanor and Mitchison 1990; Uzawa et al. 1990). The cellular amount of the Rum1 protein is strictly regulated in a cell cycle-dependent manner such that it is extremely low in exponentially growing cells, whereas it is accumulated in those arresting at G1. Thus, the fluctuation of the Rum1 protein amount ensures the activation of the Cdc2/Cdc13 kinase at the G1/M boundary and inhibition of premature entry into M phase from the G1 phase (Kelly et al. 1993; Nishitani and Nurse 1995). It is, therefore, crucial to know how the level of Rum1 is regulated in the cell cycle. Molecular mechanisms on regulation of the level of the Rum1 protein, however, remain to be determined, except that it is known that it is not transcriptionally regulated. The amount of rum1+ mRNA remains constant between exponentially growing cells and G1-arrested cells. This result suggests that the level of Rum1 protein may be regulated through protein turnover.

It is known that the level of cdc18+ mRNA as well as that of the protein is cell cycle-dependent, peaking at the G1/S boundary (Kelly et al. 1993; Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). Cdc18, which shows amino acid sequence similarity to budding yeast Cdc6 (Zhou et al. 1989), is crucial for the initiation of S phase and regulates coupling between Start and S phase and also between S phase and M phase. To initiate DNA replication at the correct timing, the Cdc18 protein level must be high at the G1/S boundary, whereas to restrain rereplication at other phases of the cell cycle, the synthesized protein must somehow be inactivated after S phase. To achieve such a cell cycle-dependent fluctuation, the Cdc18 protein would be expected to be short-lived and indeed it is shown to be (Muzi-Falconi et al. 1996). However, no molecular analysis has been addressed on the regulation of stability of the Cdc18 protein.

In the present study, we have identified the pop1+ gene, which is important for maintenance of ploidy; mutations of this gene result in appearance of diploid or polyploid cells. We have found that the Rum1 and Cdc18 proteins are highly accumulated in the pop1 mutant, which accounts for increased ploidy of the pop1 mutant. The pop1+ gene encodes a member of a WD-repeat family with the F-box motif that is involved in the ubiquitin-dependent proteolysis (Bai et al. 1996). We will show that the Rum1 and Cdc18 proteins are degraded through the proteasome pathway. Rum1 and Cdc18 are polyubiquitinated in the proteasome mutant, but not in the pop1 mutant. Finally Pop1 forms a complex with Cdc18 in vivo. These findings show that the WD-repeat protein Pop1 is a novel regulator of the cell cycle that is required for degradation of Rum1 and Cdc18 through the ubiquitin-proteasome pathway.

Results
pop1 mutations lead to an increased ploidy and cell size
Using flow cytometry (FACS), during the course of searching for sterile mutants because of a failure to arrest at G1 phase upon nutrient starvation we isolated a novel type of mutant that showed increased ploidy. Wild-type cells arrest at a 1C content of DNA under nitrogen starvation condition, which is prerequisite for conjugation (Fig. 1A). In contrast, 14 mutants showed no G1 peak; instead, they exhibited increased ploidy. In particular, three mutants (364, 422, and 577) possessed a higher amount of DNA content such as 4C, 8C, and possibly more in a discrete manner (Fig. 1 B–D, left), whereas the other 11 mutants showed 2C and 4C [data not shown]. Also the forward scattering pattern that reflects cell size showed that, in proportion to the increase in DNA content, the cell size of these mutant cells was also increased (Fig. 1 A–D, right). Genetic analysis indicated that all the three mutants were recessive and allelic, designated pop1+ (polyploid).

FACS analysis in the exponentially growing pop1 mutant showed a similar pattern to that seen in nitrogen-starved condition. Examples of cell morphology of the pop1 mutant stained with DAPI are shown in Figure 1E. Not only cell length but also cell width of these mutants is clearly larger than that of wild type. In addition, a single large nucleus was apparent, suggesting that onset of mitosis rather than cytokinesis is defective in the pop1 mutant. The cell size of the pop1-364 mutant doubles on average compared with wild-type haploid
cells, whereas that of diploid cells shows an ~50% increase, consistent with the fact that some population of the pop1 mutant contains more than 4C DNA content (Fig. 1B). The generation time of the pop1 mutant is 30% longer than that of wild-type cells.

Figure 1. Phenotypes of the pop1 mutants. [A-D] Cells [A, wild type, TP114-2A; B, 364; C, 422; D, 577] were grown in low nitrogen medium for 24 hr and processed for flow cytometry. [Left, x-axis] DNA content of individual cells in a logarithmic scale and [y-axis] frequency. [Right, x-axis] Forward scattering and [y-axis] DNA content. [E] Wild type (left) or pop1-364 (right) cells were grown in rich YPD medium and stained with DAPI. Bar, 10 μm.

pop1 mutants are supersensitive to K-252a, a drug that causes over-replication

To analyze genetically defective phenotypes of pop1, the following experiments were performed. It has been reported that a drug K-252a causes overreplication when added to fission yeast cells as well as animal culture cells (Usui et al. 1991, 1995). Thus, sensitivity to K-252a was examined. It was found that the pop1 mutant is supersensitive to K-252a (Table 1) but not to a related compound staurosporine [data not shown].

The pop1 mutations show a synthetic lethal interaction with cdc13 or cdc2

Next, synthetic phenotypes were examined between pop1 mutants and those in cdc2 or cdc13. The pop1 mutations (-364, -577) were synthetically lethal when combined with temperature-sensitive cdc13-117; dissection of 22 ascis yielded no viable double mutants. We, however, sometimes obtained tiny colonies of double mutants after tetrad analysis, which were unable to propagate. Cells from these abortive colonies were extremely elongated with a single large nucleus, resembling cdc13-deficient cells (Hayles et al. 1994). Conversely, multicopy plasmids containing the cdc13+ gene were capable of suppressing partially the pop1 mutation [data not shown]. These results suggest that either activity or level of the Cdc2/Cdc13 kinase complexes is decreased in the pop1 mutant.

A similar synthetic phenotype was also observed between pop1 and temperature-sensitive cdc2-33. A pop1 cdc2-33 mutant was not lethal at low temperature (26°C); however, the double mutant could not form colonies at 32°C at which either single mutant was capable of forming colonies. This result also supports the notion that the Cdc2/Cdc13 kinase was reduced in the pop1 mutant. In contrast, the pop1 mutant did not show synthetic lethal phenotypes with the G1-arrest cdc10-i29 or G2-arrest cdc25-22 mutant.

The Ruml and Cdc18 proteins are accumulated in the pop1 mutant

As a first step in analyzing defective phenotypes of the cells, whereas that of diploid cells shows an ~50% increase, consistent with the fact that some population of the pop1 mutant contains more than 4C DNA content (Fig. 1B). The generation time of the pop1 mutant is 30% longer than that of wild-type cells.

Table 1. K-252a supersensitivity of the pop1 mutant

| Strains | Concentrations of K-252a (μg/ml) |
|---------|---------------------------------|
|          | 0     | 6      | 10     | 20    |
| Wild type| ++    | ++     | +      | -     |
| pop1-364 | ++    | +      | -      | -     |
| Δpop1    | ++    | +      | -      | -     |
| Δpck2    | ++    | ++     | -      | -     |

Each strain was streaked on rich YPD plates with or without indicated concentrations of K-252a and incubated at 30°C for 3 days.

*pop1-364 (364); Δpop1 (KK203); and Δpck2 (TP170-2B).

[+], [-] Colony size was indistinguishable from that on plates without drug, [+] smaller colonies were formed, [-] no colonies were formed.
pop1 mutant at a molecular level, the amount of various cell cycle regulators was examined by immunoblotting. Proteins examined include Cdc2, Cdc13, Cdc18, and Rum1. Total cell extracts were prepared from wild-type, the pop1 mutant, Δrum1, and rum1+ overproducing cells, and immunoblotting was performed using respective antibodies. As shown in Figure 2A, it was found that the level of Rum1 protein was increased significantly in the pop1 mutant (lane 2). As reported previously (Correa-Bordes and Nurse 1995), the level of Rum1 protein in exponentially growing wild-type cells was extremely low and hardly detected (lane 1). In contrast, cells carrying ectopically overexpressed rum1+ gene on plasmids produced a high level of Rum1 protein (lane 4).

In addition to the Rum1 protein, the level of Cdc18 was also found to be accumulated in the pop1 mutant (Fig. 2B, lane 2). In contrast to an increased level of Rum1 and Cdc18, the level of Cdc13 and Cdc2 was not changed significantly in the pop1 mutant (Fig. 2C,D; tubulin was used as a loading control in Fig. 2E).

Accumulation of the Rum1 protein in the pop1 mutant is not attributable to an increased transcription

To address the question of how the Rum1 protein is accumulated in the pop1 mutant, total RNAs were prepared from the same strains as used in immunoblotting and Northern analysis was performed with the rum1+ gene as a probe. As shown in Figure 2F, in a clear contrast to the protein level, the amount of the rum1+ mRNA was not increased in the pop1 mutant (cf. lanes 1 and 2), whereas a significant increase in the rum1+ RNA was detected in cells where the rum1+ gene was expressed ectopically (lane 4; note that the difference in the size of the rum1+ mRNA because of ectopic expression). These results suggest that the increase in Rum1 protein in the pop1 mutant is not attributable to transcriptional induction of the rum1+ gene; instead, it is a result of post-transcriptional or post-translational mechanisms (see below).

The pop1 mutant is more susceptible to a higher dosage of the rum1+ or cdc18+ gene

If the accumulation of the Rum1 and/or Cdc18 proteins is responsible for the increased ploidy in the pop1 mutant, it is predicted that even moderate overexpression of the rum1+ or cdc18+ gene in the pop1 mutant would be lethal, whereas wild-type cells would be able to survive. To examine this possibility, the pop1 mutant was transformed with multicopy plasmids that contain the rum1+ or cdc18+ gene under the thiamine-repressible nmt1 promoter of either the strong (pREP3X) or crippled version (pREP41 and 81). We found that the pop1 mutant carrying either pREP3X–rum1+ or pREP41–rum1+ grew extremely slowly compared with those carrying an empty vector even in repressed condition (Fig. 3A, left). In wild type, pREP3X–rum1+, not pREP41–rum1+, is toxic only in derepressed condition (Fig. 3A, middle). This result indicates that the pop1 mutant becomes highly susceptible to an increase of the rum1+ gene dosage possibly because of the increase in Rum1 protein level.

An analogous susceptibility to an increased gene dosage was also observed in the cdc18+ gene. The pop1 mutant carrying pREP41–cdc18+ was capable of forming tiny colonies on plates in derepressed conditions, whereas wild-type cells carrying pREP41–cdc18+ could form colonies of size indistinguishable from those carrying a vector [Fig. 3A, left, and 3B, left and middle]. Similarly, wild-type cells carrying pREP3X–cdc18+ managed to form tiny colonies, whereas no colonies were formed in the pop1 mutant carrying pREP3X–cdc18+. Thus pop1 becomes susceptible to an increased gene dosage of either rum1+ or cdc18+ consistent with the accumulation of Rum1 and Cdc18 in the pop1 mutant.

Figure 2. The Rum1 and Cdc18 proteins are accumulated in the pop1 mutant. (A–E) Wild type (lane 1), the pop1 mutant (lane 2, KK203), Δrum1 (lane 3), or wild type containing pREP3X–rum1+ (lane 4) was grown either in rich YPD liquid medium (lanes 1–3) or in minimal medium (induced for 16 hr, lane 4). Total proteins (25 μg) were run in each lane. (F,G) Total RNAs were prepared from the same strains as in A–E. Labeled probes containing the rum1+ [F] or his3+ [G] genes were used for hybridization.
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**Figure 3.** Genetic interaction between pop1 and rum1 or cdc18. (A,B) Wild-type or pop1 mutant (KK203) carrying a vector, pREP3X-rum1+ or pREP41-rum1+ [A], or pREP3X-cdc18+, pREP41-cdc18+, or pREP81-cdc18+ [B] was streaked on minimal plates with [left] or without [middle] thiamine. (C,D) Δrum1 or pop1 rum1 mutant carrying a vector [C] or pREP3X-rum1+ [D] was grown on minimal medium containing thiamine. Note that Δrum1 cells containing pREP3X-rum1+ [D, left] were sporulating. Bar, 10 μm.

Rum1 is required for the polyploid phenotype of the pop1 mutant

To examine a consequence of the loss of the rum1+ gene function in the pop1 mutant, a double mutant between pop1 and Δrum1 was constructed. It was found that the polyploid phenotype caused by the pop1 mutation was suppressed, although not completely [see below] by the rum1 deletion; cell size of the pop1 rum1 double mutant was much smaller than that of the pop1 mutant [Fig. 3C, right; see Fig. 1E for comparison]. FACS analysis indicated that the double mutant contains the 2C content of DNA like wild type [data not shown]. The double mutant is still sterile like the Δrum1 cells. Introduction of the rum1+ gene into this double mutant reverted to wider and elongated cell morphology, reminiscent of the pop1 mutants [Fig. 3D, right]. This result demonstrates that increased ploidy in the pop1 mutant is attributable to the accumulation of a CDK inhibitor, Rum1. It should be noted that cell size of the pop1 rum1 double mutant is still slightly longer than that of either wild type or Δrum1 [Fig. 3C, cf. right and left panels]. This phenomenon led us to pursue a relationship between Pop1 and Cdc18.

The Cdc18 protein accumulates in the pop1 mutant independently of the Rum1 function

It has been reported that cells carrying an increased dosage of the rum1+ gene accumulate the Cdc18 protein, although the molecular mechanisms remain to be determined [Jallepalli and Kelly 1996; Fig. 2B, lane 4]. It is, therefore, interesting to see whether the Cdc18 protein still accumulates in the pop1 rum1 double mutant. If the accumulation of Cdc18 in the pop1 mutant is a secondary effect attributable to the accumulation of Rum1, the pop1 rum1 double mutant would contain a normal amount of Cdc18. If, on the other hand, the amount of the Cdc18 protein is regulated by Pop1 independently of Rum1, it would be expected that Cdc18 still accumulates in the double mutant. Immunoblotting indicated that the latter is the case. The double mutant still contained a higher amount of Cdc18, like a single pop1 mutant or cells containing ectopically expressed rum1+ gene [Fig. 4A]. This result shows that the accumulation of Cdc18 in the pop1 mutant is not a secondary effect attributable to the accumulation of Rum1 and that Pop1 regulates the amount of the Rum1 and Cdc18 proteins in an independent manner.

In line with the independence of the accumulation of Rum1 and Cdc18 in the pop1 mutant, it was found that, like in a pop1 single mutant [Fig. 3B], pREP41–cdc18+ as well as pREP3X–cdc18+ is toxic in the pop1 rum1 double mutant [Fig. 4B]. Cells of the pop1 rum1 double mutant carrying pREP41–cdc18+ became much longer, whereas wild-type cells carrying the same plasmid did not [Fig. 4C]. These results support further the notion that Cdc18 accumulates in the pop1 mutant in the absence of Rum1 and that the accumulation of Cdc18 does contribute phenotypes of the pop1 mutant, although minor compared with that of Rum1.

The predicted Pop1 protein belongs to a WD-repeat protein family

The pop1+ gene was isolated by complementation using cdc2-33 pop1-364 double mutant as a host [see Materials and Methods]. A 2325-bp uninterrupted open reading frame [ORF] that encodes a protein of 775 amino acid residues was found [Fig. 5A]. A comparison of the predicted amino acid sequence between Pop1 and known proteins shows that the Pop1 protein is a member of WD-repeat proteins, which are composed of tandem repeats of the unit consisting of (GH-X_{13,41}–WD)^{N4–8}
The Pop1 protein appears to contain seven repeats. A homology search against known databases (EMBL, PIR, and SWISS-PROT) indicates that the Pop1 protein shows the highest homology to Cdc4 from budding yeast Saccharomyces cerevisiae (Fong et al. 1986) and its putative homolog from Candida albicans (GenBank accession no. X96763). Overall identity among these proteins is not extremely high (30% between Pop1 and ScCdc4 and 35% between Pop1 and CaCdc4). It is, however, noteworthy that not only central WD-repeat motifs but also both amino- and carboxy-terminal flanking regions show a significant homology (Fig. 5B). These flanking regions of the Pop1 protein do not show any homology to other members of WD-repeat proteins. In particular, Pop1 contains the F-box motif, which is reported recently to exist in various proteins including Cdc4 and implicated in ubiquitin-mediated proteolysis (Fig. 5B,C; Bai et al. 1996). Functional exchangeability between Pop1 and ScCdc4 has been performed; however, no positive results have been obtained (K. Kominami and T. Toda, unpubl.).

Figure 4. Stability of the Cdc18 protein in the pop1 mutant is regulated independently of Rum1. (A) Wild type (lane 1), the pop1 mutant (KK203, lane 2), Δrum1 [lane 3], pop1 rum1 [lane 4], or wild type containing pREP3X-rum1* [lane 5] was grown as described in Fig. 2. (B) Wild type or the pop1 rum1 double mutant carrying indicated plasmids was streaked on minimal plates with [left] or without [middle] thiamine. (C) Morphology of cells carrying pREP41-cdc18* grown on minimal plates is shown. [Left] Wild type; [right] the pop1 rum1 mutant. Bar, 10 μm.

Gene disruption of the pop1* gene

The pop1* gene was disrupted by one-step gene replacement method (Fig. 5A; see Materials and Methods). The pop1-deleted Δpop1 cells were viable and essentially the same phenotypes as the original mutants observed: increased ploidy and sterility. Thus, the pop1* gene is not essential for viability but required for maintenance of ploidy.

Identification of the pop1* gene product

To identify the pop1* gene product, the pop1* gene was fused with a tag and introduced into the pop1 mutant. Immunoblotting using anti-hemagglutinin (HA) antibody showed that the Pop1 protein was detected as a band of ~90 kD. Approximately 40% of the Pop1 protein was found as an insoluble fraction (Fig. 6A, lanes 5,6). This might imply that some of the Pop1 protein forms a large structural complex inside the cell.

As a first step in determining the cell cycle-dependent regulation of the level of the Pop1 protein, the amount of Pop1 during the cell cycle was examined by arrest-release using the G2-arrest cdc25 mutant. No noticeable fluctuation of the Pop1 protein in the cell cycle appeared to occur (Fig. 6B).

The Rum1 and Cdc18 proteins are accumulated in the proteasome-deficient mutant

In budding yeast Cdc4 is involved in ubiquitin-dependent proteolysis of a CDK inhibitor, Sic1 (Schwob et al. 1994). Bearing this in mind, the possibility that Rum1 and Cdc18 are degraded through the proteasome-mediated pathway was examined. To this end, the temperature-sensitive mts3-1 mutant that is defective in subunit 14 of the 26S proteasome regulatory complex (Gordon et al. 1996) was used. Total cell extracts were prepared from wild type and mts3-1, which were incubated at restrictive temperature (35.5°C) and were immunoblotted using anti-Rum1 and anti-Cdc18 antibodies. As shown in Figure 7A, it was found that both Rum1 and Cdc18 proteins became accumulated in the mts3-1 mutant at 35.5°C (lanes 5–8). It is noteworthy that even at 0 hr, the Rum1 protein could be detected in the mts3-1 mutant (lane 5). The extent of the accumulation of the Rum1 protein was higher than that of the Cdc18 protein, probably because of cell cycle-dependent transcriptional regulation of the cdc18* gene (Nishitani and Nurse 1995, Muži-Falconi et al. 1996). This result strongly suggests that the Rum1 and Cdc18 proteins are degraded through the 26S proteasome-dependent pathway and the Pop1 protein is involved in degradation of these two proteins.

The Rum1 and Cdc18 proteins are polyubiquitinated but not in the pop1 mutant

If Rum1 and Cdc18 are degraded through the 26S proteasome, it would be expected that these proteins become polyubiquitinated in the proteasome mutants. If so, it...
Figure 5. Cloning of the pop1* gene and its predicted amino acid sequence. (A) Restriction map and complementation test of the pop1* gene. Arrowhead represents a subclone where the SacI site was disrupted. Restriction enzymes shown: (BglII) BglII; (H) HindIII; (Mlu) MluI; (ScaI) ScaI; (Sma) Sma. (B) Schematic drawing of the fission yeast Pop1 (Pop1), S. cerevisiae Cdc4 (ScCdc4) and C. albicans Cdc4 homolog (CaCdc4). Values represent percentage of identity of amino acids. Note that the third WD repeat in Pop1 and CaCdc4 is shown with ovals as the number of amino acids in these repeats is larger (see Fig. 5C). (C) A comparison of amino acid sequences of Pop1, ScCdc4, and CaCdc4. Identical amino acids are shown in closed squares; conservative amino acids are shown in gray squares. The position of the WD repeats and F-box is also shown.

would be interesting to see what happens in the pop1 mutant. To examine this possibility, a tagged version of Rum1 (Rum1–HA) was overproduced in the mts3-1 or pop1 mutant and ubiquitination of Rum1 was examined (see Materials and Methods). Immunoblotting with anti-HA antibody against extracts prepared from mts3-1 incubated at 26°C (Fig. 7B, lane 3) or 35.5°C (lane 2) revealed that, in addition to a predicted size of Rum1-HA (38 kD), higher molecular mass forms were detected in a discrete pattern. To show ubiquitination of Rum1-HA, the same filter was immunoblotted with anti-ubiquitin antibody. As expected, a similar, if not identical, band pattern of the higher molecular mass forms (emphasized with horizontal bars) was detected with anti-ubiquitin antibody (lanes 5, 6). It should be noted that anti-ubiquitin antibody did not recognize a band of 38 kD, which is nonubiquitinated, showing specificity of anti-ubiquitin antibody. The ubiquitinated forms of Rum1-HA were more abundant in the mts3-1 mutants incubated at the restrictive temperature (35.5°C, lane 5) than at the permissive temperature (26°C, lane 6). This result demonstrates clearly that Rum1 is polyubiquitinated and degraded by the ubiquitin-26S proteasome pathway.

In contrast to a pattern in the mts3-1 mutant shown above, no higher molecular mass forms of Rum1-HA were detected, and, more important, no ubiquitinated bands were present in the pop1 mutant (Fig. 7B, lanes 1, 4). Control immunoblotting with anti-Cdc2 antibody showed a similar level of the Cdc2 proteins with no higher molecular mass forms (Fig. 7B, bottom).

In addition to Rum1, similar results were obtained in Cdc18; myc-Cdc18 was polyubiquitinated in the mts3-1 mutant, but not in the pop1 mutant (Fig. 7C). These results establish the notion that Pop1 is required for ubiquitination and degradation of Rum1 and Cdc18.

The Pop1 protein forms a complex with Cdc18

To define the biochemical function of the Pop1 protein more precisely, the possibility of physical interaction between Pop1 and Cdc18 was examined. Cell extracts were prepared from cells that expressed HA-tagged Pop1 (Pop1–HA) and myc-tagged Cdc18 (myc–Cdc18) (Fig. 8, lane 1), and immunoprecipitation was performed with anti-HA antibody in the presence or absence of competi-
A total ppt sup

**Figure 6.** Identification of the Pop1 gene product. (A) Cells carrying a vector (lanes 1, 4, 7) or plasmids containing the tagged pop1÷ gene (lanes 2, 3, 5, 6, 8, 9) were grown in minimal medium. Total extracts (lanes 1–3) were fractionated into insoluble (lanes 4–6) and soluble (lanes 7–9) fractions. (B) Exponentially growing cdc25 mutant carrying the tagged pop1÷ gene was first arrested at 35.5°C for 4 hr and 15 min and shifted back to 26°C. Aliquots were taken every 15 min. Percentage of separated cells is indicated to show synchrony. cdc25 carrying an empty vector was shown at the left (cont).

tors. The Cdc18 protein, both tagged (70 kD) and endogenous (65 kD), was coprecipitated with Pop1-HA (Fig. 8, lane 2). Neither Pop1 nor Cdc18 was precipitated when immunoprecipitation was performed in the presence of excess HA peptide (lane 3), demonstrating that the interaction between Pop1 and Cdc18 is specific. This result suggests that Pop1 functions to recognize and bind protein substrates leading to ubiquitination and degradation through the proteasome machinery.

**Discussion**

In this study we have identified a novel cell cycle regulator, Pop1, as a critical determinant for the maintenance of genome ploidy. In a pop1 mutant the CDK inhibitor Rum1 and the S-phase regulator Cdc18 accumulate to high level. In contrast, the pop1 rum1 double mutant no longer shows increased ploidy, suggesting that the pop1 mutant phenotype is a result of the accumulation of the Rum1 protein. We have shown that the Rum1 and Cdc18 proteins are accumulated in the mutant defective in one of the subunits of the 26S proteasome. Moreover, these proteins contain higher molecular mass polyubiquitinated forms with discrete band patterns. In contrast, in the pop1 mutant, no ubiquitinated forms of these proteins were detected. Finally, we have shown that Pop1 binds Cdc18 in vivo. The Pop1 protein might, therefore,
We have shown that Rum1 protein is accumulated in the ploidy mutants. As shown in this study, we isolated the lethal mutants to be insufficient to drive successive S phase, as the amount of Cdc18 protein found in the mutants is insufficient to inhibit mitoses completely. In addition, the amount of Cdc18 protein accumulated in the popl mutant and might need to be degraded for meiotic progression. Alternatively, it is possible that in the popl mutant the overreplication occurs only in some cell cycles at a frequency that does not affect the ability to obtain colonies. Because the popl mutants we have isolated are not conditional, at present it is difficult to estimate precisely the frequency of rereplication per cell cycle in the mutant.

**Targets of Pop1-dependent proteolysis: Rum1 and Cdc18**

We suspected initially that a sole target of Pop1-dependent proteolysis might be Rum1 as the defective phenotypes of the pop1 rum1 double mutant are modest; FACS analysis indicated a normal ploidy (2C). However, subsequent analyses using the pop1 rum1 double mutant have convinced us that the accumulation of the two proteins in the popl mutant is a parallel process. Popl regulates protein stability of Rum1 and Cdc18 in an independent manner.

Regarding targets for Popl, we cannot conclude definitely that Rum1 and Cdc18 are the only proteins regulated by Popl. As shown here, the high ploidy phenotypes are suppressed in the popl rum1 double mutants. We have found several other phenotypes of the pop1 mutant in addition to increased ploidy, including a low viability in stationary phase, supersensitivity to UV exposure, and a high rate of chromosome loss. The former two phenotypes are also suppressed by the rum1 disruption [K. Kominami and T. Toda, unpubl.], suggesting that the major targets of Pop1-dependent proteolysis are Rum1 and Cdc18.

**Proteasome, ubiquitin, and Pop1**

Our data show that Rum1 and Cdc18 are degraded through the proteasome-dependent pathway. It is interesting to point out that, in line with this notion, mts3-1 mutant cells have been reported to rereplicate their DNA at restrictive temperature [Gordon et al. 1996]. Although it was interpreted previously that overreplication is not a result of a bypass of M phase, phenotypic analysis of polyplody in the mts3-1 mutant needs to be reexamined in view of the accumulation of Rum1 and Cdc18. On the other hand, in the mts2-1 mutant, which is defective in subunit 4 of the 26S proteasome [Gordon et al. 1993], neither Rum1 nor Cdc18 is accumulated [K. Kominami and T. Toda, unpubl.]. It is, therefore, possible that Rum1 and Cdc18 are degraded by proteasome in a Pop1-dependent and 26S subunit-specific manner.

We have shown that Pop1 forms a complex with Cdc18. This may suggest that a primary function of Pop1 is to recognize and bind specific substrates that are then ubiquitinated and targeted to proteasome for degradation. This is consistent with the results, which show that no ubiquitination of Rum1 and Cdc18 occurs in the pop1 mutant. Pop1 may act as a tether between substrates and the E2/E3 complexes, or Pop1 may be a component of E3. Recent analysis shows that budding yeast Clns and Sic1 become phosphorylated before ubiquitination and degradation [Lanker et al. 1996; Schneider et al. 1997].
CDK inhibitors Rum1 and Sic1 and possibly S-phase regulators Cdc18 and Cdc6. An interesting question then is whether fission yeast has CDK inhibitors other than Rum1 that inhibit Cdc2/Cig1,2 or Puc1, another G1-type cyclin [Forsburg and Nurse 1991].

A further analogy can be seen in mammalian systems where a CDK inhibitor, p27Kip1, has been shown to be degraded through ubiquitin-dependent proteolysis [Pagano et al. 1995]. Interestingly, it has been shown that Ubc3 (and Ubc2) is responsible for degradation of p27Kip1. In animal cells, CDK inhibitors that inhibit specifically Cdc2/cyclin A or B have not been identified. There is, however, circumstantial evidence that supports the existence of such CDK inhibitors [e.g., Waldman et al. 1996]. In opposition to the current view of the cellular function of CDK inhibitors [Sherr and Roberts 1995], the phenotypes of the pop1 mutant leading to Rum1 and Cdc18 accumulating are apparently analogous to those of tumor cells, which also result in increased ploidy, genome instability, and failure to differentiate [Hartwell and Kastan 1994]. Identification of novel CDK inhibitors as well as a Pop1 homolog in mammalian cells would be a future issue to explore evolutionarily conserved functions of a WD-repeat protein.

Materials and methods

Strains, media, genetic techniques, and nomenclatures

The Schizosaccharomyces pombe strains used in this study are listed in Table 2. HM123 was used as wild type unless otherwise stated. Standard procedures for S. pombe genetics were followed according to Moreno et al. [1991]. Low nitrogen minimal medium [0.25% NH₄Cl instead of 0.5%] was used for nitrogen starvation experiments. Plates contained 1.6% agar. All the cultures were grown at 30°C unless otherwise stated. Gene disruptions are abbreviated as the gene preceded by Δ, such as Δpop1.

Isolation of the pop1 mutants

h₃⁶ strain (TP114-2A, Table 1) was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine. Sterile mutants [1008] were first isolated by iodine vapor treatment from ~200,000 mutagenized colonies. Each sterile mutant was grown in low nitrogen medium for 3 days at 26°C and processed for FACS as described previously [Kumada et al. 1995].

Allelism tests

A cross between each mutant and wild type or a pairwise cross among mutants was performed using protoplast fusion method [Moreno et al. 1991]. Crosses between the pop1 mutants [364, 422, and 577] and wild type [HM143] showed that a mutation in the three mutants is recessive and segregates as a single chromosomal locus. In addition, all the three mutants [KK200, 422, and 577] did not complement each other, demonstrating that they were derived from mutations in the same locus.

Nucleic acids preparation and manipulation

Standard molecular biology techniques were followed as described [Sambrook et al. 1989]. Enzymes were used as recommended by the suppliers [New England Biolabs Co.]. For Northern analysis, [γ-³²P]ATP was used to label DNA probes [a 693-bp
Table 2. Fission yeast strains used in this study

| Strain       | Genotype      | Derivations                                      |
|--------------|---------------|--------------------------------------------------|
| HM123        | h'·leu1       | our stock                                        |
| HM143        | h'·ade6·M210  | our stock                                        |
| PN513        | h'·leu1·ura4  | P. Nurse [ICRF, London, UK]                      |
| TP114-2A     | h'·leu1·ura4  | our stock                                        |
| TP170-2B     | h'·leu1·pck2·LEU2 | this study                                        |
| 364          | h'·leu1·ura4·pop1-364 | this study                                        |
| 422          | h'·leu1·ura4·pop1-422 | this study                                        |
| 577          | h'·leu1·ura4·pop1-577 | this study                                        |
| PN1012       | h'·leu1·ura4·ade6·rum1·ura4' | P. Nurse [ICRF, London, UK]                      |
| cdc25        | h'·cdc25-22   | this study                                        |
| KK199        | h'·ura4·pop1-364 | this study                                        |
| KK200        | h'·ade6·M210·pop1-364 | this study                                        |
| KK201        | h'·leu1·pop1-364·cdc2-33 | this study                                        |
| KK202        | h'·leu1·ura4·pop1-364·rum1·ura4' | this study                                        |
| CHP428/429   | h'·leu1·leu1·ura4·ura4·his7·hid7·ade6·M210·ade6·M216 | C. Hoffman [Boston College, Chestnut Hill, MA]    |
| KK203        | h'·leu1·ura4·pop1·ura4' | this study                                        |
| mts3         | h'·leu1·mts3-1 | C. Gordon [Western General Hospital, Edinburgh, UK] |

PCR fragment containing the rum1' gene and a 1.2-kb fragment containing the his3' gene.

Construction of the rum1' or cdc18' gene under control of the thiamine-repressible nmt1 promoter
Two oligonucleotides were used to amplify the rum1' gene by PCR: 5'-GGGGATCCTTATCGTAATAAATTGTGCCCTGT-3', where the NdeI and BamHI sites are underlined. A 696-bp amplified fragment was subcloned into pREP41 (Basi et al. 1993), designated pREP41-rum1'. pREP41-rum1' is functional as it is capable of suppressing the Δrum1 disruptant. pREP3X-rum1', pREP3X-cdc18', pREP41-cdc18', pREP41-myc-cdc18', and pREP81-cdc18' were obtained from Dr. P. Nurse [ICRF].

Cloning of the pop1' gene
The pop1-364 cdc2-33 strain (KK201) was used to isolate the pop1' gene. Briefly the double mutant was transformed with an S. pombe genomic library constructed in a vector pDR248. LEU' transformants that could form colonies at 32°C but not at 36°C were selected. In total 14,000 colonies were screened and three transformants were obtained. Three plasmids that contained different but overlapping insert DNAs (pKK10, 103, and 105) were obtained.

Nucleotide sequence determination
The nucleotide sequence of a 2.8-kb Smal-BglII fragment (Fig. 5A) was determined with the dideoxy method (Sanger et al. 1977). These sequence data are available from EMBL/GenBank/DBJ under accession no. Y08391.

Gene disruption
Two kinds of plasmids were constructed, an inserton and a complete deletion. For insertion, a 1.8-kb ura4' fragment was inserted into an internal SacI site of pPK103 (Fig. 5A). For a deletion method, a 2.2-kb BglII fragment that encompasses 94% of ORF was deleted and replaced with a 1.8-kb ura4' fragment. A diploid strain (CHP428/429) was used to disrupt the pop1' gene.

Stable Ura' transformants were picked and the disruption of the pop1' gene was verified by PCR and then tetrad analysis was performed. We obtained four viable spores were uracil auxotrophy segregates 2:2 and Ura' segregants showed polyploid phenotype. The pop1-364 mutant did not complement with Δpop1, demonstrating that the gene we cloned is pop1'.

Construction and expression of the tagged pop1' and rum1' genes
The tagged version of the pop1' gene was constructed as follows. The entire pop1' gene was amplified by PCR using two oligonucleotides, 5'-GGGGATCCTTATCGTAATAAATTGTGCCCTGT-3', where the NdeI site is underlined and the 3' oligonucleotide is 5'-GGGGATCGGCCGCGTTCGTAATAAATTGTGCCCTGT-3'. For insertion, a 1.8-kb SmaI-BglII fragment was replaced with a 2.2-kb BglII fragment of pKK103, where the NdeI site is underlined. A PCR-amplified 2342-bp fragment was subcloned into pREP41 [HA-6His] (provided by Dr. J. Millar, National Institute for Medical Research, London, UK) to yield pREP41-pop1'·HA·6His, which expresses the gene under the control of a partially crippled version of the nmt1 thiamine-repressible promoter (Basi et al. 1993) and contains six His and two repeats of a peptide derived from the influenza HA. pREP41-pop1'·HA/6His was made by subcloning a 2.4-kb NdeI·BamHI fragment from pREP41-pop1'·HA/6His into pREP42. Tagging of 6His·HA in the carboxyl terminus did not interfere with the normal function of the Pop1 protein as pREP41-pop1'·HA/6His suppressed the pop1 mutation. A 2279-bp BglII-BamHI fragment containing HA/6His was replaced with a 2.2-kb BglII fragment of pPK103, where the pop1'·HA/6His was regulated under a natural promoter. The tagged version of the rum1' gene [pREP41-rum1'·HA/6His] was constructed in a manner similar to pop1'·HA/6His except that the following primers were used: 5'-GGGGATCCTTATCGTAATAAATTGTGCCCTGT-3' and 5'-GGGGATCGGCCGCGTTCGTAATAAATTGTGCCCTGT-3'.

Immunological assays
Antibodies used in this study were following, anti-Cdc2, anti-Cdc13 (provided by Dr. H. Yamano, ICRF), anti-Rum1 (affinity-purified, a gift from Dr. J. Correa-Bordes, ICRF), anti-Cdc18 [af-
prepared as follows essentially according to Watanabe et al. (1991). To detect ubiquitination of Ruml and Cdc18, cell extracts were prepared as follows: 1% SDS, 0.5 M PMSF containing 10% of trichloroacetic acid (TCA) and disrupted with glass beads. After washing with acetone five times, the proteins were solubilized by boiling (3 min) in the extraction buffer containing 1% SDS and 0.1 mM ubiquitin aldehyde (kindly provided by Dr. Keiji Tanaka, The Tokyo Metropolitan Institute of Medical Science, Japan) to prevent deubiquitination. The supernatant was mixed with an equal amount of the HB buffer (Moreno et al. 1991). Standard procedures for immunoblotting were followed (Harlow and Lane 1988) except that CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer was used to detect proteins <50 KD (Matsuda et al. 1987). For immunoprecipitation, cell extracts (2.5 mg) were mixed with anti-myc antibody or anti-HA antibody, followed by protein G-Sepharose beads (Pharmacia). HA-peptide (40 mg) was added to extracts in a competitor experiment. Anti-ubiquitin antibody was used in 1:2500 dilution. Horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad) and a chemiluminescence system (ECL, Amer sham) were used to detect bound antibody.

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