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]

Received December 5, 1996; revised version accepted April 25, 1997.

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 rerepli-

cation without intervening mitoses (Usui et al. 1991). In fly, mutations either in *Dmcdc2* 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

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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 (Deshaies 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 G₂/M boundary and inhibition of premature entry into M phase from the G₁ phase (Moreno and Nurse 1994). 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. 1B–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. 1A–D, right). Genetic analysis indicated that all the three mutants were recessive and allelic, designated *pop1* (polyploidy).

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

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 μ m.

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.

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 asci 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 *cdc10-129* or G₂-arrest *cdc25-22* mutant.

The Rum1 and Cdc18 proteins are accumulated in the pop1 mutant

As a first step in analyzing defective phenotypes of the

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

Strains ^a	Concentrations of K-252a (μ g/ml) ^b			
	0	6	10	20
Wild type	++	++	+	–
<i>pop1-364</i>	++	+	–	–
Δ <i>pop1</i>	++	+	–	–
Δ <i>pck2</i>	++	++	+	–

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

^a*pop1-364* (364); Δ *pop1* (KK203); and Δ *pck2* (TP170-2B).

^b(++) 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, $\Delta rum1$, 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).

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), $\Delta rum1$ (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 μ g) 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.

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 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) $\Delta rum1$ or *pop1 rum1* mutant carrying a vector (C) or pREP3X-*rum1*⁺ (D) was grown on minimal medium containing thiamine. Note that $\Delta rum1$ cells containing pREP3X-*rum1*⁺ (D, left) were sporulating. Bar, 10 μ m.

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 $\Delta rum1$ 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 $\Delta rum1$ 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 $\Delta rum1$ (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-X₂₃₋₄₁-WD)^{N4-8}

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), $\Delta rum1$ (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 μ m.

(Neer et al. 1994; Fig. 5B,C). 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.).

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 ($\Delta pop1$) 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; Muzi-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 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 septated 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,

Figure 7. Degradation of Rum1 and Cdc18 through the 26S proteasome and ubiquitination of Rum1 and Cdc18. (A) Wild type (lanes 1–4) or *mts3-1* (lanes 5–8) cells grown at 26°C were shifted to 35.5°C. Aliquots were taken at 0 hr (lanes 1,5), 2 hr (lanes 2,6), 4 hr (lanes 3,7), 6 hr (lanes 4,8), and immunoblotting was performed. (B) Extracts were prepared (Materials and Methods) from *mts3-1* (lanes 2,3,5,6) or *pop1* (lanes 1,4) containing pREP41-*rum1*⁺-HA/6His, which was grown in the absence of thiamine for 24 hr at 26°C and then shifted to 35.5°C for 4 hr. Immunoblotting was performed either with anti-HA antibody (lanes 1–3) or with anti-ubiquitin antibody (lanes 4–6). Overlapping bands between these two blots were emphasized with horizontal lines. Note that an abundant 38-kD nonubiquitinated form (Rum1-HA) was not detected with anti-ubiquitin antibody. (●) A band that is present in both *mts3-1* and *pop1*. This band may be nonspecific because it is not detected with anti-ubiquitin antibody. (C) A similar experiment as in B was performed to show ubiquitination of Cdc18 except that cells (*mts3-1* at 35.5°C in lanes 2 and 4, or *pop1* at 35.5°C in lanes 1 and 3) expressing pREP41-myc-*cdc18*⁺ were used. Immunoblots were performed either with anti-Cdc18 (lanes 1,2) or with anti-ubiquitin (lanes 3,4). Again, 70-kD nonubiquitinated form (myc-Cdc18) was not detected with anti-ubiquitin antibody.

Figure 8. Physical interaction between Pop1 and Cdc18. Extracts were prepared from cells (PN513) that expressed pREP42-*pop1*⁺-HA/6His and pREP41-myc-*cdc18*⁺. Immunoprecipitation was performed with anti-HA antibody in the absence (lane 2) or presence (lane 3) of HA-peptide. A pattern of immunoblots before immunoprecipitation was also shown (lane 1).

be a component or regulator of the ubiquitin-proteasome-dependent pathway that specifically recognizes a CDK inhibitor and an S-phase regulator.

Identification of *Pop1* as a critical regulator of ploidy control

Despite extensive screening for diploidizing mutants, the *pop1* mutants have not been identified before (Broek et al. 1991; Hayles et al. 1994). We reason that this is because of sterility and meiosis-deficiency of the *pop1* mutants. Previous screening was based on the assumption that diploidized mutants would undergo meiosis and form spores, which was used to enrich such increased ploidy mutants. As shown in this study, we isolated the *pop1* mutants from our collections of sterile mutants using FACS. Sterility of the *pop1* mutants could be attributable to inability of the *pop1* mutant to arrest at G₁ upon nitrogen starvation, which is prerequisite for conjugation (Kumada et al. 1995). Failure of meiosis might be related to the accumulation of Rum1 in the *pop1* mutant, which would block the meiotic activation of Cdc2/Cdc13 complexes. Alternatively, proteins other than Rum1 and Cdc18 may become accumulated in the *pop1* mutant and might need to be degraded for meiotic progression.

The nonlethal phenotype of the *pop1* disruptant is not surprising, as it has been reported that fission yeast cells can tolerate being pentaploid (Molnar and Sipiczki 1993). We have shown that Rum1 protein is accumulated in the *pop1* mutant, but the amount is far less than that found in lethal *rum1*⁺-overproducing cells where the *rum1*⁺ gene is expressed ectopically. The amount of Rum1 protein accumulated in the *pop1* mutant would be insufficient to inhibit mitoses completely. In addition, the amount of Cdc18 protein found in the *pop1* mutant appears to be insufficient to drive successive S phase, as the *pop1 rum1* double mutant no longer shows a high ploidy phenotype. We suspect that only some proportion of the *pop1* cells bypass M phase and rereplicate in each cell cycle. Alternatively, it is possible that in the *pop1* mu-

tant the overreplication occurs only in some cell cycles at a frequency that does not affect the ability to obtain colonies. Because the *pop1* 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 *pop1* mutant is a parallel process. Pop1 regulates protein stability of Rum1 and Cdc18 in an independent manner.

Regarding targets for Pop1, we cannot conclude definitively that Rum1 and Cdc18 are the only proteins regulated by Pop1. As shown here, the high ploidy phenotypes are suppressed in the *pop1 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 polyploidization 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.

1996; Willems et al. 1996). Cdc18 and Rum1 might need to be phosphorylated in order for Pop1 to recognize and bind.

Similarity to other systems: Budding yeast Cdc4, Sic1, and Cdc6, and human p27^{Kip1}

It is intriguing that in budding yeast Cdc4 is also involved in degradation of a CDK inhibitor, Sic1, through ubiquitin-mediated proteolysis. In the temperature-sensitive *cdc4* or *cdc34* mutant that is defective in ubiquitin-conjugating enzyme Ubc3 (Goebel et al. 1988), the Sic1 protein as well as Cdc6 accumulates (Schwob et al. 1994; Piatti et al. 1996). In parallel with Rum1, Sic1 becomes stabilized in G₁, which prevents premature activation of Cdc28/B-type cyclins (Clb1-6) complexes. Furthermore, like the *pop1* mutant, overproduction of the B-type cyclin Clb4 is capable of suppressing partially the temperature-sensitive *cdc4-1* mutant (Bai et al. 1994). Although overall homology between Pop1 and Cdc4 is not extremely high (30%), it is worth pointing out that Cdc4 is a protein that shows the highest homology in the entire budding yeast genome. In addition, the F-box motif that appears to be required for a physical interaction between Cdc4 and Skp1 is also conserved in Pop1. Thus it is very likely that Pop1 is a fission yeast homolog of Cdc4. Skp1 has been shown to be also required for ubiquitin-mediated proteolysis of Cln2, Clb5, and Sic1 (Bai et al. 1996). Cdc34 (Ubc3), Cdc53, and Skp1 are shown to act closely with Cdc4 and Sic1 (Schwob et al. 1994; Mathias et al. 1996; King et al. 1996). It is interesting to see whether in fission yeast these homologs function with Pop1 to regulate Rum1 and Cdc18.

In spite of the parallels between Pop1–Rum1 and Cdc4–Sic1, defective phenotypes of the *pop1* and *cdc4* mutants look very different. For example, the *CDC4* gene is essential and mutant cells arrest in G₁ with multiple buds, whereas *pop1*⁺ is not essential and the mutant leads to overreplication. In addition, in budding yeast, mutants in subunits of the anaphase promoting complex (APC) give rise to polyploidy, implying that this complex is involved in degrading proteins that results in polyploidy when stabilized (Heichman and Roberts 1996). This phenotypic difference, however, could be explained in the following manner. Sic1 inhibits Cdc28 kinase activities associated with all six B-type cyclins (Clb1–6) responsible for an S-phase progression (Donovan et al. 1994; Nugroho and Mendenhall 1994; Schwob et al. 1994). As Cln cyclins are stabilized in the *cdc34* mutant (Deshaies et al. 1995; Blondel and Mann 1996), the *cdc4* mutant cells continue budding without DNA replication. In contrast, Rum1 inhibits Cdc2/Cdc13 and partially Cdc2/Cig2, but not Cdc2/Cig1 (Correa-Bordes and Nurse 1995; Fisher and Nurse 1996; Martin-Castellanos et al. 1996). Thus even though a high level of Rum1 is present, S phase still occurs owing to activities of Cdc2/Cig1,2, which can compensate for loss of the S-phase function of B cyclins (Fisher and Nurse 1996; Martin-Castellanos et al. 1996). A common function of Pop1 and Cdc4 can, therefore, be summarized as degradation of

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, p27^{Kip1}, 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 p27^{Kip1}. 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 Δ*pop1*.

Isolation of the pop1 mutants

*h*⁹⁰ 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	<i>h⁻leu1</i>	our stock
HM143	<i>h⁹⁰ade6-M210</i>	our stock
PN513	<i>h⁻leu1 ura4</i>	P. Nurse (ICRF, London, UK)
TP114-2A	<i>h⁹⁰leu1 ura4</i>	our stock
TP170-2B	<i>h⁻leu1 pck2::LEU2</i>	our stock
364	<i>h⁹⁰leu1 ura4 pop1-364</i>	this study
422	<i>h⁹⁰leu1 ura4 pop1-422</i>	this study
577	<i>h⁹⁰leu1 ura4 pop1-577</i>	this study
PN1012	<i>h⁹⁰leu1 ura4 ade6 rum1::ura4⁺</i>	P. Nurse (ICRF, London, UK)
cdc25	<i>h⁺cdc25-22</i>	P. Nurse (ICRF, London, UK)
KK199	<i>h⁹⁰ura4 pop1-364</i>	this study
KK200	<i>h⁹⁰ade6-M210 pop1-364</i>	this study
KK201	<i>h⁹⁰leu1 pop1-364 cdc2-33</i>	this study
KK202	<i>h⁹⁰leu1 ura4 pop1-364 rum1::ura4⁺</i>	this study
CHP428/429	<i>h⁻h⁺leu1 leu1 ura4 ura4 his7 hid7 ade6-M210 ade6-M216</i>	C. Hoffman (Boston College, Chestnut Hill, MA)
KK203	<i>h⁻leu1 ura4 pop1::ura4⁺</i>	this study
mts3	<i>h⁻leu1 mts3-1</i>	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'-GGGCATATGGAACCTTCAACACCAC-3' and 5'-GGGGGATCCTTATCGTAATAAATTGTGCC-3' where *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 $\Delta rum1$ 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 pDB248. 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 (pKK101, 103, and 105) were obtained.

Nucleotide sequence determination

The nucleotide sequence of a 2.8-kb *SmaI*-*BglIII* 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 insertion and a complete deletion. For insertion, a 1.8-kb *ura4⁺* fragment was inserted in an internal *SacI* site of pKK103 (Fig. 5A). For a deletion method, a 2.2-kb *BglIII* 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 where uracil auxotrophy segregates 2:2 and Ura⁺ segregants showed polyploid phenotype. The *pop1-364* mutant did not complement with $\Delta pop1$, 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; the 5' oligonucleotide is 5'-GGGCATATGAATGAGAAGAAAAAGA-3' where the *NdeI* site is underlined and the 3' oligonucleotide is 5'-GGGGCGGCCGC-TAGAAGATTTAGTAGATCCAA-3' where the *NotI* 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. pREP42-*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 *BglIII*-*BamHI* fragment containing HA/6His was replaced with a 2.2-kb *BglIII* fragment of pKK103, 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'-GGGCATATGGAACCTTCAACACCAC-3' and 5'-GGGGGGGGCGGCCGCTTCGTAATAAATTGTGCCTGT-3'.

Immunochemical 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-

finitly-purified, a gift from Dr. H. Nishitani, ICRF), anti-tubulin antibody (T-5168, Sigma), anti-HA (16B12, BAbCO), anti-myc (9E10, ICRF), and anti-ubiquitin monoclonal antibody (MAB1510, Chemicon Inc.). Fission yeast cell extracts were prepared as described (Moreno et al. 1991). To detect the Rum1 protein in total cell extracts, cells were boiled for 5 min before breakage with glass beads (Correa-Bordes and Nurse 1995). To detect ubiquitination of Rum1 and Cdc18, cell extracts were prepared as follows essentially according to Watanabe et al. (1997). Cells were rapidly killed by addition of the ice-cold extraction buffer (50 mM Tris-HCl at pH 7.4, 1 mM EDTA, 10 mM β -mercaptoethanol, 0.5 mM 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 μ M 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 (Matsudaira 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 μ g) 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, Amersham) were used to detect bound antibody.

Acknowledgments

We thank Drs. J. Correa-Bordes, C. Gordon, C. Hoffman, J. Millar, H. Nishitani, P. Nurse, and H. Yamano for the gifts of strains, plasmids, and antibodies and H. Nakano for K-252a. Special thanks are given to J. Correa-Bordes and Y. Watanabe, who instructed preparation of cell extracts and immunoblotting; to B. Baum, who kindly performed Northern blotting; and to K. Tanaka, who generously gave us anti-ubiquitin antibody and ubiquitin aldehyde. We are grateful to D. Hirata, who first suggested the cloning of the *pop1*⁺ gene with complementation using the *pop1 cdc2-33* double mutant as a host; K. Nasmyth, for stimulative discussion and comments on the manuscript; and S. Moreno, for communicating results prior to publication. We thank P. Nurse and J.F.X. Diffley for useful comments and critical reading of the manuscript. This work was in part supported by Kyowa Hakko Co.

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Genes Dev. 1997, **11**:

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