

# The *Schizosaccharomyces pombe* *cdc3*<sup>+</sup> Gene Encodes a Profilin Essential for Cytokinesis

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**Abstract.** The fission yeast *Schizosaccharomyces pombe* divides by medial fission and, like many higher eukaryotic cells, requires the function of an F-actin contractile ring for cytokinesis. In *S. pombe*, a class of *cdc*<sup>-</sup> mutants defective for cytokinesis, but not for DNA replication, mitosis, or septum synthesis, have been identified. In this paper, we present the characterization of one of these mutants, *cdc3*-124. Temperature shift experiments reveal that mutants in *cdc3* are incapable of forming an F-actin contractile ring. We have molecularly cloned *cdc3* and used the *cdc3*<sup>+</sup> genomic DNA to create a strain carrying a *cdc3* null mutation by homologous recombination in vivo. Cells bearing a *cdc3*-null allele are inviable. They arrest the cell cycle at cytokinesis without forming a contractile ring. DNA sequence analysis of the *cdc3*<sup>+</sup> gene reveals

that it encodes profilin, an actin-monomer-binding protein. In light of recent studies with profilins, we propose that Cdc3-profilin plays an essential role in cytokinesis by catalyzing the formation of the F-actin contractile ring. Consistent with this proposal are our observations that Cdc3-profilin localizes to the medial region of the cell where the F-actin contractile ring forms, and that it is essential for F-actin ring formation. Cells overproducing Cdc3-profilin become elongated, dumbbell shaped, and arrest at cytokinesis without any detectable F-actin staining. This effect of Cdc3-profilin overproduction is relieved by introduction of a multicopy plasmid carrying the actin encoding gene, *act1*<sup>+</sup>. We attribute these effects to potential sequestration of actin monomers by profilin, when present in excess.

**T**HE three major landmark events in the eukaryotic cell cycle are (a) replication of the genetic material that occurs in S phase; (b) partitioning of the replicated DNA to the daughter nuclei during M phase; and (c) division of the cell itself to produce two daughter cells as a result of cytokinesis. The last 10 yr have seen tremendous advances in our understanding of the molecular nature of processes occurring during the S and M phases and the controls that regulate entry into S and M phases. By contrast, the mechanisms and regulations that govern the third major landmark event, cytokinesis, remain only poorly understood.

Much of the progress in our understanding of the controls that regulate S and M phases stem from genetic studies in the fission yeast *Schizosaccharomyces pombe*. Several lines of evidence suggest that this yeast should also be an ideal system with which to study cytokinesis. First, *S. pombe* cells divide by medial fission (Nurse, 1985) and, like many higher eukaryotic cells, produce equally sized daughter cells after cytokinesis. Second, cytokinesis in fission yeast, akin to

higher eukaryotic cells, uses the function of an F-actin contractile ring (Marks and Hyams, 1985; Jochová et al., 1991). Third, conditionally lethal mutants that are defective for cytokinesis have been isolated from screens designed to identify mutants defective for cell cycle progression. These mutants uncouple the nuclear events of the cycle from cytokinesis so that under nonpermissive conditions, they accumulate multiple nuclei but do not undergo cytokinesis (Nurse et al., 1976).

Mutants defective for cytokinesis have been classified into two major categories, early cell plate mutants and late cell plate mutants. Under restrictive temperature conditions, early cell plate mutants, defined by genetic loci *cdc7*, *cdc11*, *cdc14*, and *cdc15*, arrest without detectable septum material after accumulating 8–16 nuclei (Nurse et al., 1976). Molecular and genetic analyses suggest that these genes might regulate some early aspect of cytokinesis (Marks et al., 1992; Fankhauser and Simanis, 1993). Under restrictive temperature conditions, the late cell plate mutants, *cdc3*, *cdc4*, *cdc8*, and *cdc12*, become elongated and dumbbell shaped, accumulate up to eight nuclei, and assemble ill-formed septa. Ultrastructural studies have suggested that the products of these genes might be required for normal F-actin function (Streiblová et al., 1984), a proposal supported by the characterization of one member of the late cell plate category, *cdc8*

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(Balasubramanian et al., 1992). The *cdc8<sup>+</sup>* gene has been cloned and its product has been identified as a novel tropomyosin essential for F-actin contractile ring function (Balasubramanian et al., 1992).

In this paper, we describe the molecular cloning and characterization of another member of the late cell plate category, *cdc3<sup>+</sup>*. We find that the *cdc3<sup>+</sup>* gene encodes a profilin. Profilins were identified initially as actin-monomer sequestering proteins (Markey et al., 1981). Subsequent analyses, however, have shown that the functions of profilin are more complex. Profilins seem to perform a variety of functions including actin-monomer sequestration (Markey et al., 1981), actin filament formation (Pantaloni and Carlier, 1993; Haarer et al., 1990; Cooley et al., 1992) and growth factor-mediated signal transduction to the actin cytoskeleton (Machesky and Pollard, 1993). In *S. pombe* Cdc3-profilin is required for formation of an F-actin contractile ring. When overproduced, however, Cdc3-profilin causes the loss of all detectable F-actin structures. These experiments provide in vivo evidence for the duality of profilin function described earlier from biochemical studies of profilins.

## Materials and Methods

### Strains, Media, Cell Synchronization, and Genetic Methods

*Schizosaccharomyces pombe* strains used in this study and their origins have been summarized in Table I. Media for growth of yeast cells and general yeast genetic methods were as described by Moreno et al. (1991). (Repression of transcription from *nmt1* promoter (Maundrell, 1989) was achieved by addition of thiamine to a concentration of 2  $\mu$ M.

### Yeast Transformation and Cloning by Complementation

DNA-mediated transformation of yeast cells for the purposes of cloning the *cdc3<sup>+</sup>* genomic DNA and *cdc3<sup>+</sup>* cDNA were performed by the spheroplast and the lithium acetate methods, respectively, as described by Moreno et al. (1991). Other *S. pombe* transformations were carried out by electroporation (Prentice, 1992). The *cdc3<sup>+</sup>* genomic DNA was cloned as follows. An *S. pombe* genomic library constructed in the plasmid vector pUR19 (Barbet et al., 1992), which carries the fission yeast *ura4* gene as a selectable marker, was a generous gift of Dr. A. M. Carr (Medical Research Council, Cell Mutation Unit, University of Sussex, United Kingdom). This library was introduced into *cdc3-124 ura4-D18 h<sup>-</sup>* (KGY435) cells, and transformants were selected on Edinburgh minimal medium plates lacking uracil containing 1.2 M sorbitol at 25°C. To identify those transformants that carried plasmids capable of reversing the temperature-sensitive lethal pheno-

Table I. *S. pombe* Strain List

Strain	Genotype	Source
KGY28	972 <i>h<sup>-</sup></i>	P. Nurse*
KGY161	<i>cdc3-124 h<sup>-</sup></i>	P. Nurse
KGY246	<i>ade6-M210 ura4-D18 leu1-32 h<sup>-</sup></i>	P. Nurse
KGY247	<i>ade6-M210 ura4-D18 leu1-32 h<sup>+</sup></i>	P. Nurse
KGY248	<i>ade6-M216 ura4-D18 leu1-32 h<sup>-</sup></i>	P. Nurse
KGY433	<i>cdc3-124 leu1-32 h<sup>-</sup></i>	This study
KGY435	<i>cdc3-124 ura4-D18 h<sup>-</sup></i>	This study
MBY34	KGY247/KGY248	This study
MBY37	<i>cdc3::ura4/cdc3<sup>+</sup></i> KGY247/KGY248	This study
MBY39	KGY247 LEU2 <i>pnmt1::cdc3</i> cDNA	This study
MBY40	<i>cdc3::ura4/cdc3-124 KGY247/KGY248</i>	This study
MBY62	<i>cdc3::ura4 ade6-ura4-D18 leu1-32 pMB301</i>	This study

\* P. Nurse is affiliated with the Imperial Cancer Research Fund (London).

type of KGY435 cells, transformants were replica plated to EMM plates lacking uracil and held at 36°C. 12 colonies in which the rescue of *cdc3-124* mutation was mitotically unstable were identified. Plasmid DNA was isolated from 10 of these strains by digestion of cell walls with zymolyase and further purified with glass milk as described (Moreno et al., 1991). Recovered plasmid DNA was used to transform *Escherichia coli* MC1061 cells, which were selected for resistance to ampicillin, and confirmed to be capable of rescuing *cdc3-124*. The *cdc3<sup>+</sup>* cDNA was cloned by complementation of the temperature-sensitive lethality of a *cdc3-124 leu1-32 h<sup>-</sup>* (KGY433) strain. An *S. pombe* cDNA expression library under control of the *nmt1* promoter in plasmid REP3X, a modified version of pREP3 (Maundrell, 1993), was a generous gift of Drs. B. Edgar and C. Norbury. Plasmid REP3X carries the budding yeast *LEU2* gene, which complements *S. pombe leu1-32* mutants, as a selectable marker. This cDNA library was introduced into KGY433 cells, and selection was applied directly for leucine prototrophy, as well as growth under restrictive temperature conditions. 32 colonies meeting these criteria were obtained. Subsequent analysis confirmed that all of these plasmids carried cDNAs representing the *cdc3<sup>+</sup>* locus.

### Molecular Biology Methods

Standard techniques for DNA manipulation and bacterial transformations were used (Sambrook et al., 1989). The *cdc3<sup>+</sup>* gene was sequenced as follows. Subclones derived from the 1-kb *SacI*-*Bam*HI fragment, which was previously determined to carry the *cdc3-124* rescuing activity, were created in phagemid vectors pTZ18R and pTZ19R (Pharmacia, Uppsala, Sweden). Single-stranded DNAs corresponding to these subclones were generated, and nucleotide sequences were determined using Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's instruction. The SwissProt database was searched using the FASTDB program (Brutlag et al., 1990) with the predicted amino acid sequence of Cdc3p as the query sequence.

### Molecular Cloning of the *S. cerevisiae* PFYI Gene

Complementary DNA corresponding to the budding yeast gene encoding profilin was amplified by polymerase chain reaction from an *S. cerevisiae* cDNA library constructed in the plasmid pRS316 (Liu et al., 1992). This library was kindly provided to us by Dr. Anthony Weil. Primers were designed based on the published sequence of the budding yeast *PFYI* gene (Magdolen et al., 1988). The cleavage site for the restriction enzyme *Bam*HI was also included in each primer to facilitate cloning of the *PFYI* gene as a *Bam*HI fragment. The amplified product was cloned into pSK(+) (Stratagene, La Jolla, CA), and its sequence was verified. Subsequently *PFYI* was moved as a *Bam*HI-*Bam*HI fragment into the *S. pombe* expression vector pREP4 (Maundrell, 1993) to produce plasmid pMB310.

### Deletion of the *Cdc3* gene

Plasmid MB309 was created by deleting the *Cla*I-*Bam*HI fragment carrying all but the NH<sub>2</sub>-terminal four amino acids from the predicted *cdc3<sup>+</sup>* coding region (see Fig. 2 B) and replacing it with a 1.8-kb fragment carrying the *S. pombe ura4<sup>+</sup>* gene. pMB309 was linearized by digestion with *Kpn*I and *Sph*I, and a 4.7-kb fragment carrying the *cdc3::ura4<sup>+</sup>* DNA was purified after agarose gel electrophoresis. This linear fragment was used to transform the uracil auxotrophic diploid strain MBY34, and selection was applied for colony formation on agar plates lacking uracil. Transformants were replica plated five times at 1-d intervals to media containing uracil to allow loss of any autonomously replicating DNA molecules carrying the *ura4<sup>+</sup>* gene. Cells were subsequently replica plated to medium lacking uracil, and clones that still remained prototrophic for uracil were treated as putative stable integrants. Genomic Southern blot analysis and analysis of spores confirmed that one of these strains was heterozygous at the *cdc3* locus of the genotype *cdc3<sup>+</sup>/cdc3::ura4<sup>+</sup>*.

### Expression in *E. coli*

Cdc3p was expressed in *E. coli* as two different fusion proteins. The full-length *cdc3<sup>+</sup>* cDNA was amplified by polymerase chain reaction using oligonucleotides 5'-TTGCAAAAATGCTCTGG-3' and 5'-TTGTCATGTCAAATCT-3'. The amplified product was treated with Klenow enzyme to produce blunt ends and inserted into pTZ19R at the *Sma*I site. Subsequently, *cdc3<sup>+</sup>* cDNA was moved as a *Bam*HI-*Eco*RI fragment into the *E. coli* expression vectors pGEX2T (Smith and Johnson, 1988) and pRSETA (Invi-

trogen, San Diego, CA) to produce plasmids pKG251 and pKG233, which expressed Cdc3p as a fusion with glutathione S-transferase (GST)<sup>1</sup> and a polyhistidine track (His<sub>6</sub>) containing peptide respectively. Pilot experiments showed that both fusion proteins, GST-Cdc3p and His<sub>6</sub>-Cdc3p, were synthesized at high levels and were soluble in aqueous solutions. The GST-Cdc3p fusion protein was purified over a glutathione agarose affinity column, and the His<sub>6</sub>-Cdc3p fusion protein was purified taking advantage of the high affinity of His<sub>6</sub> to Ni<sup>2+</sup>.

### Antibodies

Polyclonal antisera were raised in two different rabbits (FLOrence and CLEOpatra) as follows. In each case, 750 µg of soluble His<sub>6</sub>-Cdc3p fusion protein was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into these rabbits. Booster injections with 100 µg of fusion protein emulsified in Freund's incomplete adjuvant were administered at monthly intervals. Antibodies specific to Cdc3-profilin were purified from an antigen affinity column. GST-Cdc3p fusion protein was covalently coupled to cyanogen bromide-activated Sepharose beads and packed in a column. Serum from each rabbit was passed through this column to allow binding of antibodies specific to Cdc3-profilin to the affinity matrix. After washing the column extensively, antibodies bound to the affinity matrix were eluted in fractions into tubes containing 0.1 M Tris, pH 8.0, with 0.15 M glycine, pH 2.5. The purity of the eluted fractions was assessed by immunoblotting. Fractions containing Cdc3-profilin-specific antibodies were pooled. The antibody concentration was <1 µg/ml. To this pool, fetal calf serum was added to 0.1% wt/vol final concentration, and aliquots were frozen at -70°C. Immunoglobulins from the preimmune sera were purified on a protein A-Sepharose column as described in Harlow and Lane (1988). They were used for immunofluorescence at 1 µg/ml final concentration.

### Protein and Immunoblot Analyses

Proteins were isolated from cells by disrupting cell walls with glass beads and extracting the released proteins with phosphate-buffered saline containing 1% NP-40, 2 mM EDTA, and protease inhibitors. Unless otherwise specified, proteins were separated on 6–20% polyacrylamide gradient gels containing SDS. After electrophoresis, the separated proteins were transferred by electroblotting to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). These blots were challenged with affinity-purified antiserum FLO at 1:400 dilution, incubated with <sup>125</sup>I-labeled protein A, and specific reactions were detected by autoradiography.

### Fluorescence Microscopy

Calcofluor/DAPI staining was accomplished as follows. Cells from a midlog culture were washed twice with cold PBS, resuspended in PBS containing Calcofluor at a final concentration of 50 µg/ml, and incubated on ice for 30 min. Cells were then washed three times with PBS, resuspended in PBS to give a thick suspension, and attached to poly-L-lysine-coated coverslips. These coverslips were inverted onto DAPI mounting solution carrying an antifade and visualized using an axioscope (Carl Zeiss, Inc., Thornwood, NY) and the appropriate set of light filters. Normally, staining of nuclei and cell wall material are visualized simultaneously although staining of nuclei is substantially masked by the bright fluorescence emanating from excitation of calcofluor. To facilitate clear visualization of nuclei and cell wall material, photographs were first taken immediately after mounting for calcofluor-stained images and after passage of a few minutes for DAPI-stained images, since fluorescence emitted from calcofluor was found to fade rapidly. Staining of F-actin with rhodamine-conjugated phalloidin was performed as described (Marks and Hyams, 1985). For immunostaining, cells were fixed with a mixture of formaldehyde and glutaraldehyde (Moreno et al., 1991) and stained with affinity-purified anti-Cdc3 antibodies or preimmune immunoglobulins followed by a Texas red-conjugated goat anti-rabbit IgG secondary antibody. In all cases photographs were taken using a camera mounted on the microscope with Tri-X pan 400 ASA film (Eastman Kodak Co., Rochester, NY) and printed on Agfa Rapitone PI-4 paper.

1. *Abbreviations used in this paper:* DAPI, 4',6-diamidino-2-phenylindole; GST, glutathione-S-transferase; His<sub>6</sub>, polyhistidine track.

## Results

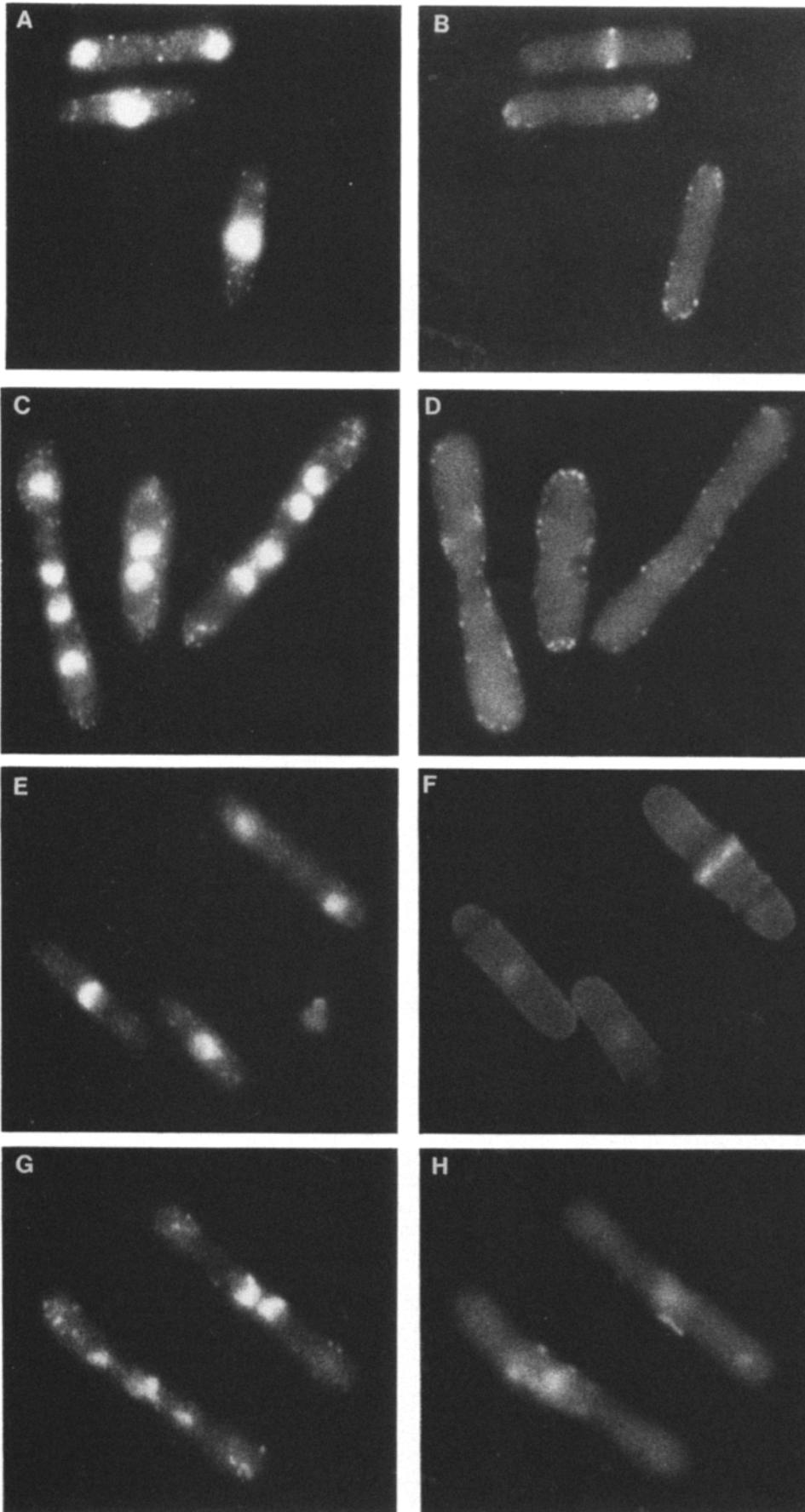
### Characterization of the *cdc3-124* Mutant

At 25°C, *cdc3-124* cells grow and divide to form colonies and are phenotypically indistinguishable from wild-type cells. At 36°C, however, the cycle is arrested and cells die after assuming an elongated dumbbell-shaped morphology, accumulating up to eight nuclei and dispersed septum material (Nurse et al., 1976; Streiblová et al., 1984). DNA replication, mitosis, and the synthesis of septum material are all unaffected in *cdc3-124* cells. To gain further insight into the nature of *cdc3-124* defect, we examined the intracellular distribution of F-actin in these cells. In wild-type cells, F-actin is observed in two distinct distributions. In growing cells, F-actin is visualized as dots and patches throughout the cell with highest concentrations at the growing end(s) (Fig. 1 B). As cells advance into mitosis, cell elongation ceases, the dot and patch staining decreases significantly, and an F-actin contractile ring forms in the medial plane of the cell (see cell with two nuclei in Fig. 1, A and B) (Jochová et al., 1991). *cdc3<sup>ts</sup>* cells arrested at the restrictive temperature for 4 h showed a markedly different distribution of F-actin (Fig. 1 D) and septum material (Fig. 1 H) (Marks et al., 1987). F-actin rings were not observed in these cells, although the dot and patch staining was not severely affected (Fig. 1 D). Since the F-actin contractile ring precedes formation of the medial septum and has been proposed to guide positioning of the septum (Marks and Hyams, 1985), this result explains why septum material in *cdc3* mutants is delocalized (Fig. 1 H) (Nurse et al., 1976; Streiblová et al., 1984). The lack of a detectable F-actin ring taken together with the delocalization of the septum material suggests that the *cdc3-124* mutants are defective for some aspect of F-actin contractile ring function.

### Molecular Cloning and Sequence Analysis

An *S. pombe* genomic library constructed in the plasmid vector pURI9 (Barbet et al., 1992) was used to isolate the *cdc3<sup>+</sup>* gene by complementation of the *cdc3-124* mutation. Three distinct plasmids rescued the *cdc3-124* mutation, and restriction enzyme analysis revealed they carried overlapping inserts (Fig. 2 A). A DNA probe derived from clone 16 (Fig. 2 A) hybridized to the NotI fragment H, PI clone 13F1p, and cosmid 29H2c in ordered *S. pombe* genomic DNA libraries (data not shown) (Maier et al., 1992; Hoheisel et al., 1993). This agrees well with the genetic location of *cdc3* (Lennon and Lehrach, 1992). Based on this and additional reasons presented in the next section, we conclude that the cloned gene is *cdc3<sup>+</sup>*, and not a high copy suppressor of the *cdc3-124* mutation.

The nucleotide sequence of a 1-kb DNA fragment (Fig. 2 A) capable of *cdc3-124* rescue was determined (Fig. 2 B). Inspection of this sequence led to the identification of a region capable of encoding a 127-amino-acid polypeptide present in three predicted exons. Perfect 5' and 3' intron consensus sequences (Russell, 1989) were found in both introns. However, branch point consensus sequences were seen only in intron 1 (Russell, 1989). To confirm the existence and position of the two introns, cDNA clones corresponding to the predicted coding region in Fig. 2 B were isolated, and their



**Figure 1.** F-actin and septum material staining in *cdc3-124* mutants. Cells were grown at 25°C to midlog growth phase and the culture was split in two. One half was shifted to 36°C, and the other half was allowed to continue growth at 25°C. After 4 h, cells were fixed and stained for F-actin with rhodamine-conjugated phalloidin or for septum material with calcofluor. In all cases, DAPI was included to visualize the nuclei. (A) DAPI staining of cells grown at 25°C; (B) F-actin staining in the cells shown in A; (C) DAPI staining in cells arrested at 36°C; (D) F-actin staining of cells in C; (E) DAPI staining in cells grown at 25°C; (F) septum staining of cells in E; (G) DAPI staining in cells arrested at 36°C; (H) septum staining of cells in G.



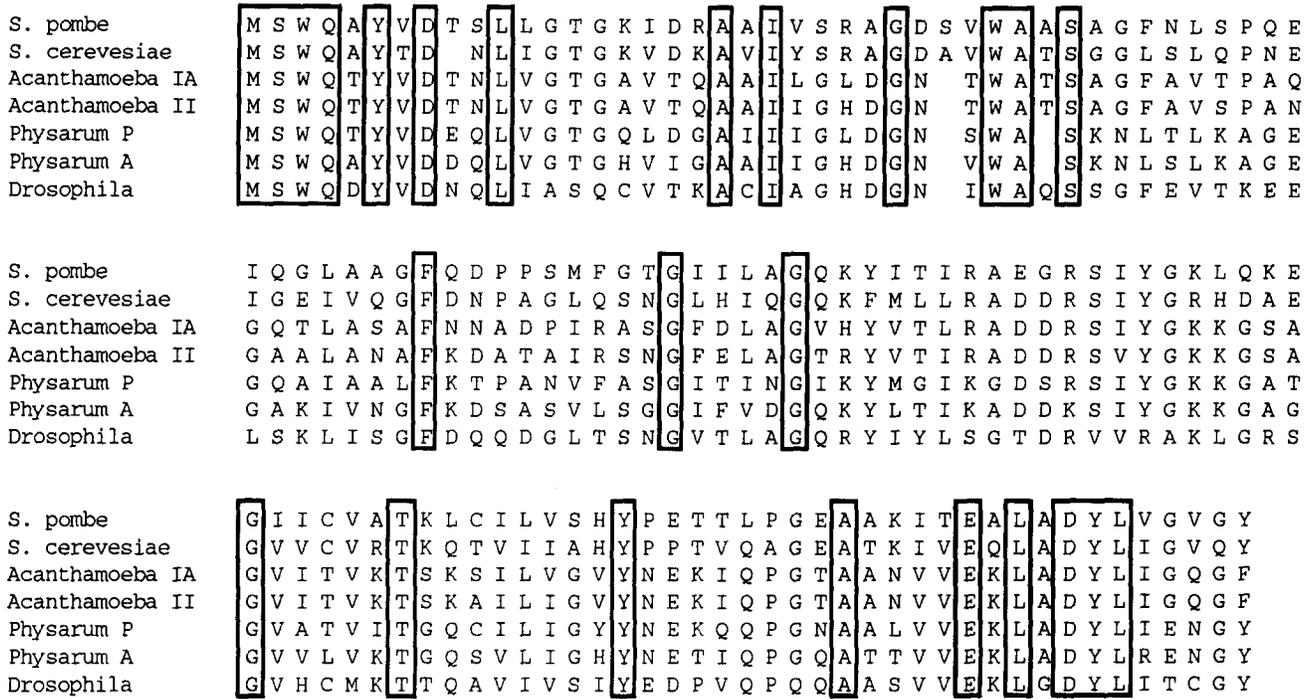


Figure 3. Alignment between the predicted amino acid sequence of Cdc3p and other profilins. Amino acids identical in all profilins have been boxed. All profilins considered in this figure, with the exception of Cdc3p, are 125 or 126 amino acids in length. Therefore, gaps have been introduced in these protein sequences to maximize alignment. See text for references to the various profilin sequences.

To test if *cdc3-124* and *cdc3::ura4* are mutant alleles of the same gene, a diploid strain of the genotype *cdc3::ura4<sup>+</sup>/cdc3-124* (MBY40) was constructed. MBY40 became temperature sensitive for colony formation at 36°C, whereas the

otherwise isogenic strain, MBY37, whose genotype differed only at the *cdc3* locus, was not. Analysis of spore products of MBY37 and MBY40 showed that in both cases, all viable colony forming spores were Ura<sup>-</sup>. Furthermore, all viable

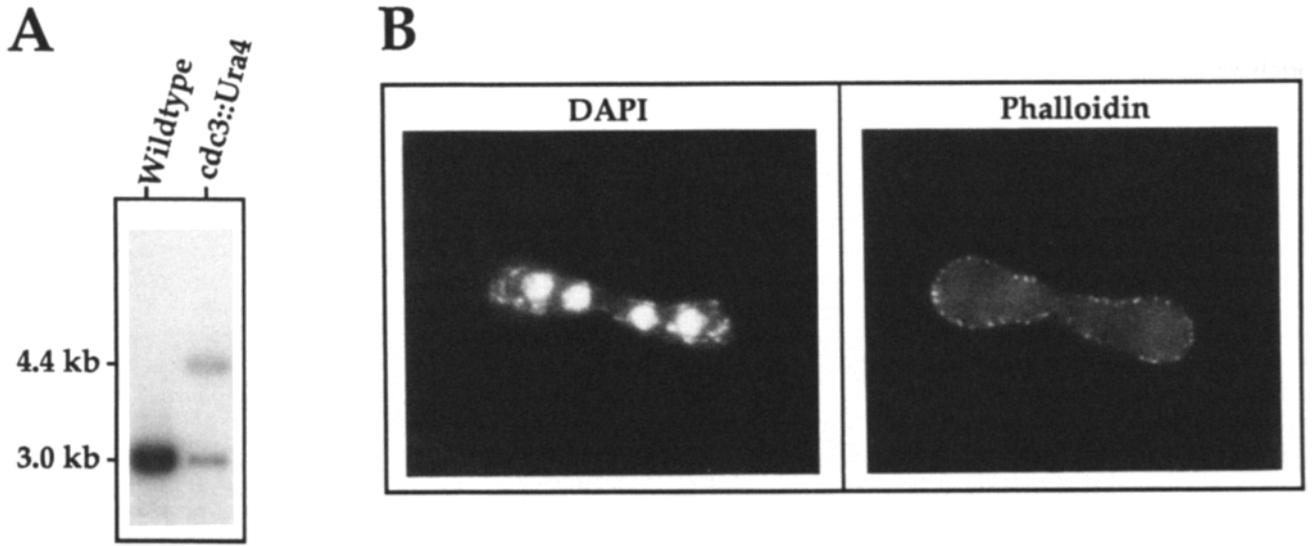
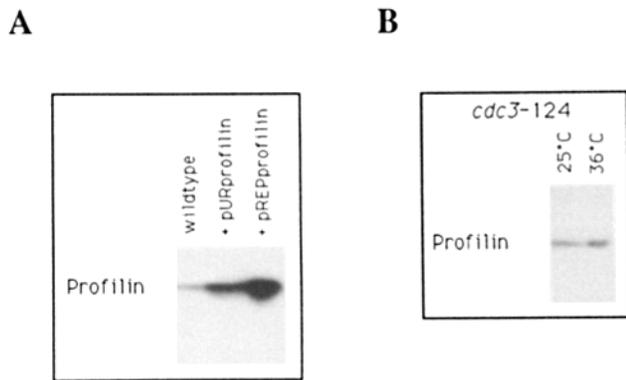


Figure 4. Analysis of a *cdc3*-null mutant. (A) Genomic Southern blot analysis to confirm deletion of a copy of the *cdc3* gene. Genomic DNA was isolated from a *cdc3<sup>+</sup>/cdc3<sup>+</sup>* strain (SP34) and a stable Ura<sup>+</sup> integrant potentially of the genotype *cdc3::ura4/cdc3<sup>+</sup>* (SP37). The DNA was digested with BamHI, separated by electrophoresis, blotted, and probed with labeled *cdc3* genomic sequences outside of the region deleted in *cdc3::ura4*. The 3-kb band represents the wild-type *cdc3<sup>+</sup>* locus and the 4.4-kb band represents the *cdc3::ura4* locus. (B) F-actin staining and nuclear architecture in *cdc3::ura4* haploids. *cdc3::ura4* haploids rescued by virtue of a plasmid-borne *cdc3<sup>+</sup>* cDNA were grown under nonselective conditions to allow for loss of the plasmid, fixed, and stained with rhodamine-conjugated phalloidin and DAPI.



**Figure 5.** Detection of Cdc3-profilin in wild-type cells, cells over-producing Cdc3-profilin, and in *cdc3-124* mutants. (A) Immunoblot with anti-Cdc3p serum, FLO. Proteins were extracted from wild-type cells carrying no plasmid, carrying a multicopy plasmid containing the *cdc3* gene (pURprofilin) or carrying a plasmid capable of producing highly elevated levels of Cdc3-profilin (pREPprofilin). The extracted proteins were separated by electrophoresis, blotted on to nylon membranes, challenged with antigen-affinity purified antiserum (FLO), and autoradiograms were generated after treatment of blots with  $^{125}\text{I}$ -labeled protein A. (B) The *cdc3-124* mutation does not affect steady-state level of Cdc3-124 profilin. Proteins were extracted from *cdc3-124* cells grown at 25°C or arrested at 36°C for 4 h and treated for immunoblot analysis as described above.

spores resulting from sporulation of MBY40 were temperature sensitive for colony formation at 36°C, whereas viable spore products of MBY37 were not. These experiments confirmed that the cloned gene encoding profilin is the wild-type allele of *cdc3* and not a high dosage suppressor.

Terminal morphology of the presumed *cdc3::ura4* spores suggested that the *cdc3<sup>+</sup>* gene is not required for spore germination, since these spores elongated and arrested uniformly as elongated and dumbbell-shaped single cells (data not shown). To further analyze the effect of *cdc3* gene deletion, *cdc3<sup>+</sup>* was introduced into MBY37 on a multicopy plasmid, and the transformant was sporulated. Haploid *cdc3::ura4* cells, rescued by virtue of plasmid-borne *cdc3<sup>+</sup>* cDNA were isolated (MBY62). These cells were grown under nonselective nutritional conditions to allow loss of the autonomously replicating *cdc3<sup>+</sup>* expressing plasmid. Microscopic examination revealed that a proportion of the cells were elongated and dumbbell shaped. Nuclear staining revealed that the elongated and dumbbell-shaped cells accumulated up to eight nuclei. Staining for F-actin with rhodamine-conjugated phalloidin displayed the presence of F-actin in dots and patches but not in the medial contractile ring (Fig. 4 C). This phenotype was similar to that seen in temperature-arrested *cdc3-124* cells (Fig. 1 D). Thus, lack of *cdc3<sup>+</sup>* function results in an inability of cells to complete cytokinesis without affecting the nuclear division cell cycle.

### Detection of Cdc3-Profilin

Affinity-purified anti-Cdc3 sera recognized a single band with an apparent relative molecular mass of 13,000 on immunoblots of total proteins prepared from wild-type *S.*

*pombe* cells (Fig. 5 A). The intensity of this band increased when the level Cdc3-profilin expression was increased either by the introduction of a multicopy plasmid bearing the *cdc3<sup>+</sup>* gene or by placement of the *cdc3<sup>+</sup>* gene downstream from the strongly transcribed *nml* promoter (Maundrell, 1989) (Fig. 5 A). These observations established that the protein recognized by these antisera is the product of the *cdc3<sup>+</sup>* gene.

We then asked if the inability of *cdc3-124* cells to undergo cytokinesis is related to the instability and disappearance of the mutant protein at the nonpermissive temperature. As illustrated in Fig. 5 B, the steady-state level of Cdc3-124 mutant profilin is no different at 36°C, suggesting that the defect in *cdc3-124* cells is not caused by the loss of Cdc3-profilin, but rather by the loss of its function.

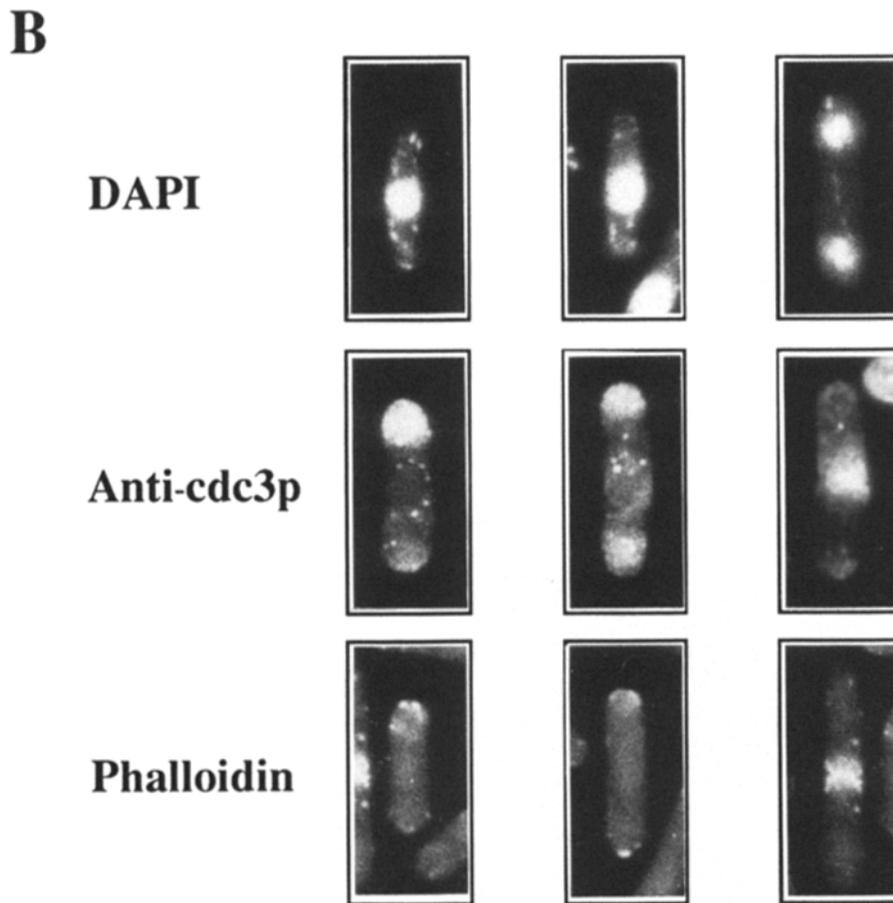
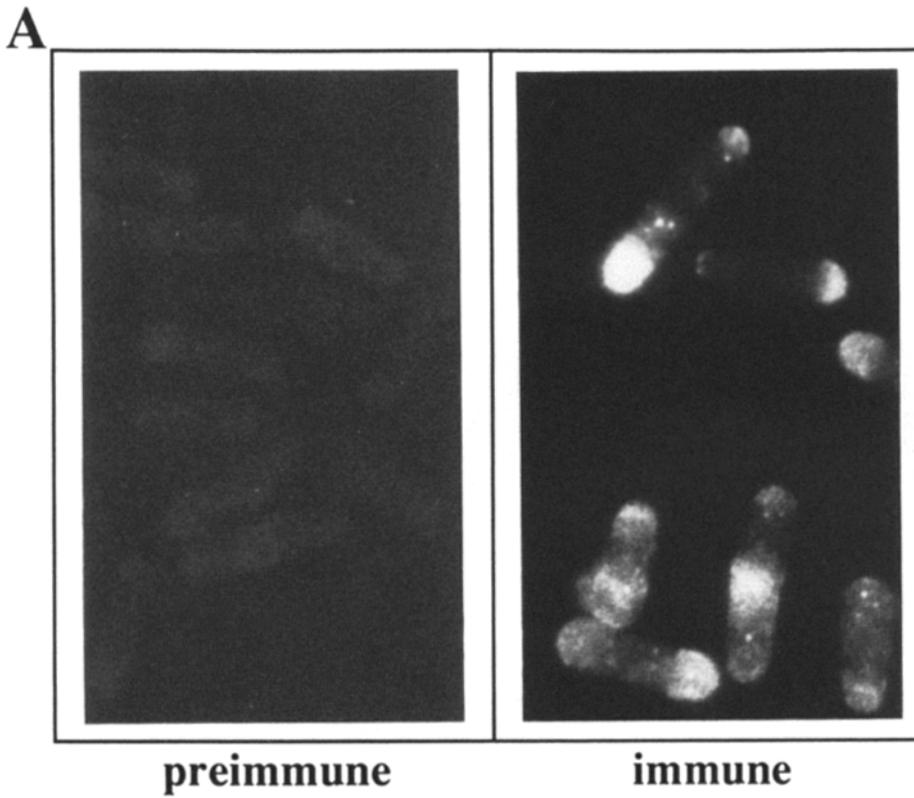
### Cdc3-Profilin Colocalizes with F-actin

Affinity-purified anti-Cdc3 sera, when used for indirect immunofluorescence, gave a strong staining pattern that was not observed when cells were stained with purified immunoglobulins from the corresponding preimmune serum (Fig. 6 A). Therefore, we were able to use these antibodies to determine the intracellular distribution of Cdc3-profilin relative to F-actin by indirect immunofluorescence.

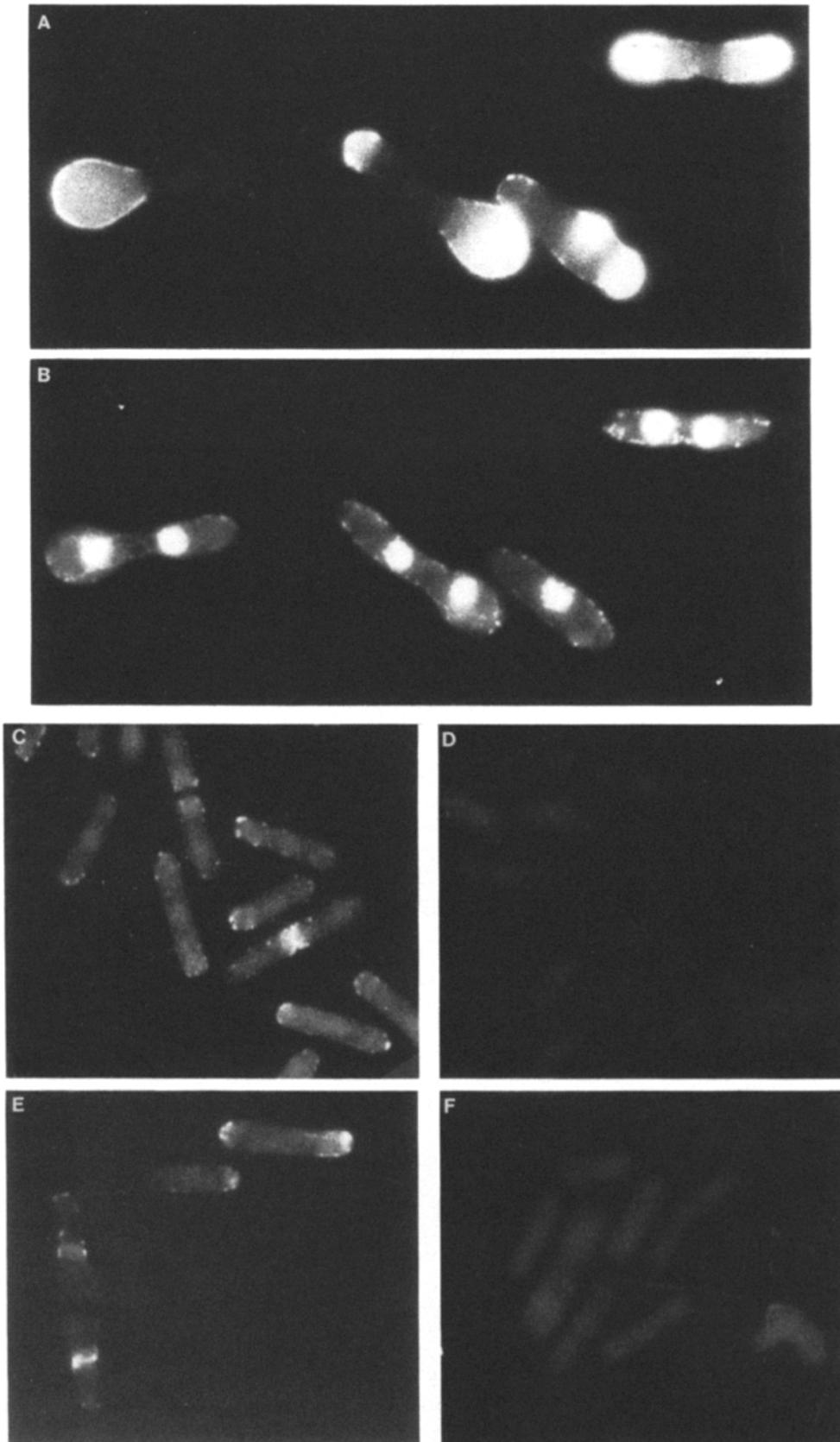
Three predominant patterns of profilin distribution were observed in wild-type cells (Fig. 6 B). In small cells, immunoreactive material was observed at one end of the cell (Fig. 6 B), presumed to be the growing end of the cell. After “new end take off” (NETO) (Mitchison and Nurse, 1985), a point in the *S. pombe* cell cycle when cells begin to grow from both poles, immunoreactive material was present at both ends of the cell. Finally, in binucleate cells, immunoreactive material was present in the medial region as a broad band. Punctate staining was observed occasionally in other regions of the cells. The nature of this staining is unknown. The locations where the vast majority of immunoreactive material was present coincided with the locations where F-actin was visualized (Fig. 6 B). However, whereas F-actin was concentrated in dots, patches, and rings, Cdc3-profilin was more diffuse in its localization.

### Overexpression of Cdc3-Profilin

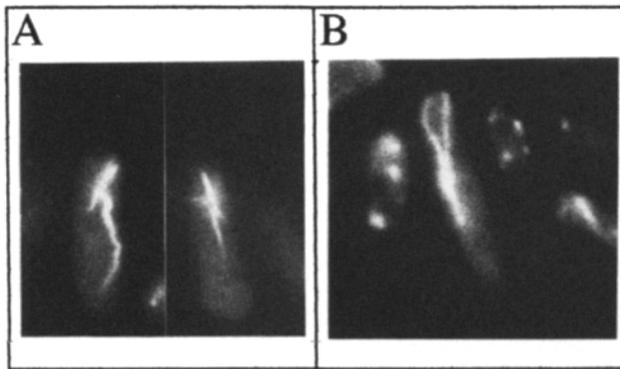
To study the effect of overproduction of Cdc3-profilin in wild-type cells, *cdc3<sup>+</sup>* cDNA was placed under control of the thiamine-repressible *nml* promoter (Maundrell, 1989), and it was integrated into the chromosome of a wild-type strain to create strain MBY39. Under conditions of transcriptional repression, MBY39 cells were phenotypically no different than wild-type cells. Removal of thiamine resulted in a 10–15-fold overproduction of Cdc3-profilin (Fig. 5 A, pREP profilin). Microscopic examination revealed that the majority of Cdc3-profilin-overproducing cells arrested as elongated and dumbbell-shaped cells, with a phenotype reminiscent of that produced by *cdc3-124* mutants at the restrictive temperature. When stained with anti-Cdc3p serum, these cells appeared intensely fluorescent, with most of the staining being present in the bulbous ends (Fig. 7, A and B). To study the correlation between localization of F-actin and Cdc3-profilin, MBY39 cells were stained with rhodamine-conjugated phalloidin. As a control, wild-type cells were stained simultaneously (Fig. 7 C). Interestingly, we ob-



*Figure 6. Cdc3-profilin and F-actin colocalize. (A) Demonstration of specificity of affinity-purified FLO antiserum. Wild-type cells were immunostained with either purified immunoglobulins from preimmune serum at 1  $\mu\text{g/ml}$  or with primary antiserum FLO at  $<1 \mu\text{g/ml}$  followed by Texas red-conjugated secondary antibodies. (B) Staining of Cdc3-profilin and F-actin in wild-type cells. Logarithmically growing wild-type cells were fixed and stained with rhodamine-conjugated phalloidin or with antiserum FLO followed by Texas red-conjugated secondary antibodies. In all cases, DAPI was also included to visualize the nuclei.*



**Figure 7.** Phenotypic analyses of cells overproducing profilins. Cells overproducing Cdc3-profilin (MBY39) were fixed and stained with anti-Cdc3p antiserum (FLO), followed by Texas red-conjugated secondary antibodies. Cells overproducing profilins or overproducing both profilin and actin were fixed and stained with rhodamine-conjugated phalloidin to visualize F-actin architecture in these cells. (A) MBY39 cells stained with anti-Cdc3p serum (FLO). (B) DAPI staining of cells in A; (C) wild-type cells stained with rhodamine-conjugated phalloidin; (D) MBY39 cells stained with rhodamine-conjugated phalloidin; (E) MBY39 cells carrying pACT7-2 stained with rhodamine-conjugated phalloidin, (F) wild-type cells overproducing *S. cerevisiae* Pfy1-profilin stained with rhodamine-conjugated phalloidin.



**Figure 8.** Phenotypic analysis of cells overproducing Cdc3-124 (E43K) mutant profilin. Wild-type (KGY246) or *cdc3-124* mutant (KGY433) cells overproducing E43K mutant profilin were fixed and stained with rhodamine-conjugated phalloidin. (A) KGY246 cells overproducing E43K mutant profilin stained with rhodamine-conjugated phalloidin; (B) KGY433 cells overproducing E43K mutant profilin stained with rhodamine-conjugated phalloidin.

served no specific fluorescent staining in cells overproducing Cdc3-profilin in the rhodamine channel (Fig. 7 D). No dots, patches, or rings were detected.

One hypothesis to explain the lack of observable F-actin structures in MBY39 is that high levels of profilin sequester actin monomers causing a deprivation of the pool of polymerization-competent actin monomers. If this was the reason for the phenotype, then it should be remedied by a compensatory overproduction of actin. To test this hypothesis, multicopy plasmids pACT7-2 and pACT9-1 carrying the *act1*<sup>+</sup> gene along with an empty control plasmid were introduced into MBY39 cells. Colonies were allowed to form first on plates containing thiamine to allow repression of transcription of the *cdc3*<sup>+</sup> gene. All plasmids transformed MBY39 cells with high efficiency, but cells carrying pACT7-2 or pACT9-1 grew slower than cells carrying the control plasmid. This can certainly be attributed to the presence of excess actin in these cells. Upon replica plating to medium lacking thiamine, we found that the cells carrying the control plasmid became elongated and dumbbell-shaped, and they displayed a phenotype characteristic of MBY39 cells as described earlier. In contrast, cells carrying either pACT7-2 or pACT9-1 continued to grow and form colonies albeit slowly. The majority of MBY39 cells carrying pACT7-2 or pACT9-1 were morphologically wild type, while the rest displayed a variety of aberrant morphologies. The distribution of F-actin in these cells was studied by staining with rhodamine-conjugated phalloidin. This analysis demonstrated that the F-actin architecture in these cells was similar to that seen in wild-type cells with staining being present in dots, patches, and rings (Fig. 7 E). Thus, overexpression of actin appeared to restore wild-type morphology and F-actin architecture to cells overproducing Cdc3-profilin.

#### **Analysis of *Saccharomyces cerevisiae* Pfy1-Profilin Function in *S. pombe***

We isolated a cDNA encoding the budding yeast Pfy1-profilin for two reasons. First, we wanted to assess the ability of Pfy1-profilin, which is most closely related to in sequence to Cdc3-profilin, to rescue the lethality of a *cdc3-124* mu-

tant. Second, we wanted to determine if overexpression of Pfy1-profilin in *S. pombe* cells produced a phenotype similar to that exhibited by cells overproducing Cdc3-profilin. To execute these experiments, the *S. cerevisiae* PFY1 cDNA was placed downstream of the *nmt1* promoter in pREP4 to produce plasmid pMB310.

Plasmid MB310 was introduced into *cdc3-124 ura4-D18* cells (KGY435), and colonies were allowed to form at 25°C in medium containing thiamine, to allow repression of transcription of the PFY1 gene. These colonies were replica plated to a range of thiamine concentrations for a 20-h period to allow for different levels of Pfy1-profilin expression, and were then shifted to restrictive temperature conditions. KGY435 cells carrying pMB310 were found to be incapable of colony formation at any level of expression. Thus, the *S. cerevisiae* Pfy1-profilin appears to be incapable of providing the essential function performed by the *S. pombe* Cdc3-profilin.

Pfy1-profilin was also overexpressed in wild-type cells to see if it had similar effects on F-actin structures, as did Cdc3-profilin. When grown under nonrepressing conditions to allow high levels of Pfy1-profilin expression, cells became elongated and dumbbell shaped, and were similar in appearance to those overexpressing the *S. pombe* Cdc3-profilin. Staining of cells carrying pMB310 with rhodamine-conjugated phalloidin failed to reveal detectable F-actin structures (Fig. 7 F).

#### **Molecular Analyses of the Cdc3-124 Mutant Profilin**

The *cdc3-124* mutant gene was cloned after amplification of the *cdc3* locus from DNA prepared from a *cdc3-124* strain. Nucleotide sequence of the *cdc3-124* gene identified a single G→A change, consistent with the mutagenic potential of nitrosoguanidine, which was used as a mutagen to isolate the *cdc3-124* mutant (Nurse et al., 1976). This G→A transition changed codon 43 in the *cdc3* coding region from GAA to AAA, resulting in a substitution of lysine for the glutamic acid residue found in this position (E43K).

To overproduce the Cdc3-124 mutant profilin, a cDNA carrying the E43K mutation was generated and cloned downstream of the *nmt1* promoter to create plasmid MB312. Plasmid MB312 was introduced into wild-type (KGY246) and *cdc3-124* (KGY433) mutant cells. Interestingly, we found that pMB312 was capable of allowing KGY433 cells to form colonies at conditions restrictive for *cdc3-124* mutants. This rescue was observed only when transcription from the *nmt1* promoter was derepressed by removal of thiamine, suggesting that an excess of the E43K mutant profilin was required for rescue of *cdc3-124* mutants. At a low level of penetrance, cell proliferation was affected in both wild-type and *cdc3-124* cells overproducing the Cdc3-124 mutant profilin, since colonies were found to contain a proportion of dead and lysed cells.

We stained KGY246 and KGY433 cells overproducing the mutant E43K mutant profilin with rhodamine-conjugated phalloidin. Surprisingly, F-actin was visualized in the form of broad bands and big patches in both wild-type (KGY246) and *cdc3-124* mutant (KGY433) cells overproducing the E43K-mutant profilin at 36°C (restrictive temperature for *cdc3-124* mutants) (Fig. 8, A and B). Such staining has not been observed in wild-type or any mutant cells studied thus far. It is therefore possible that the low penetrance lethality

in cells overproducing the mutant profilin is a consequence of the unusual F-actin structures found in these cells.

## Discussion

This study has identified the product of the *S. pombe cdc3<sup>+</sup>* gene as a profilin, a known monomeric actin binding protein. Structural and functional features that are shared between Cdc3p and two other known profilins show that the product of the *cdc3<sup>+</sup>* gene is an authentic profilin. First, Cdc3p is 53, 48, and 36% identical in sequence to the budding yeast Pfy1-profilin (Magdolen et al., 1988), the *Acanthamoeba* profilins (Pollard and Rimm, 1991) and the *Drosophila chickadee* profilin (Cooley et al., 1992) respectively. Second, expression of cDNA encoding the *Drosophila chickadee* gene completely reverses the lethality associated with loss of Cdc3-profilin, suggesting that the *Drosophila* profilin can perform all the essential functions performed by Cdc3-profilin (M. K. Balasubramanian, K. Edwards, D. Kiehart, and K. L. Gould, unpublished observations).

Why is profilin essential for cytokinesis in *S. pombe*? Analysis of the F-actin cytoskeleton of *cdc3-124* and *cdc3*-null mutants suggest that the defect in cytokinesis in these mutants results from the inability of these cells to synthesize and/or maintain a F-actin contractile ring. The contractile ring forms in the medial plane of the cell in a temporally and spatially regulated manner, being formed around midmitosis and disassembled after cytokinesis (Marks and Hyams, 1985). Although earlier biochemical studies postulated that profilin might act as an actin monomer sequestering protein (Markey et al., 1981), recent studies suggest that profilin can also function to promote actin filament assembly (Pantaloni and Carlier, 1993). It is known that ATP-bound actin monomers polymerize more rapidly than ADP-bound actin monomers, and profilin catalyzes the exchange of nucleotide on actin (Goldschmidt-Claremont, 1992). Recent studies have also shown that profilin can promote actin filament formation by lowering the critical concentration of actin (Pantaloni and Carlier, 1993). Thus, Cdc3-profilin might contribute to the formation of the F-actin contractile ring by either or both of these mechanisms. The possibility that Cdc3-profilin participates in F-actin contractile ring formation is supported by our immunolocalization experiments that showed the presence of Cdc3-profilin in the medial region of the cell where the F-actin ring will form during cytokinesis.

At least two previous studies have also provided evidence for a requirement for profilin in actin filament formation in vivo. First, budding yeast cells deleted for the profilin encoding gene *PFY1* lack F-actin cables and undergo random budding and cytokinesis (Haarer et al., 1990). Second, *Drosophila* mutants that are deficient for an oocyte specific profilin transcript are defective for cytoplasmic actin networks leading to defective intracellular cytoplasm transport and sterility (Cooley et al., 1992).

Analysis of the terminal phenotypes of *cdc3-124* and *cdc3*-null mutants illustrated that these mutants are impaired primarily for progression through cytokinesis. Spore germination, DNA replication, and mitosis were all unaffected. This presents a paradox, since it is well known that actin filament formation is required for a wide range of cellular processes.

Two possible rationales can be used to reconcile to these observations.

One possible explanation is that many components of the cytoskeleton, even in lower eukaryotes, are present as multiple isoforms that appear to perform specialized cellular functions. Although only one profilin-encoding gene has been identified in both the yeasts, multiple profilin isoforms with different biochemical properties have been found in *Acanthamoeba* and *Physarum* (Pollard and Rimm, 1991; Binnette et al., 1990). Thus, two profilin isoforms with different functions might be present in *S. pombe* cells, and the second hypothetical profilin might not be able to substitute for Cdc3-profilin in catalyzing the formation of the F-actin contractile ring.

The second explanation is that the requirement for profilin is rate limiting only for cytokinesis, and that this is the only stage in the cell cycle where the effects of its absence are apparent. In other words, the F-actin ring that forms within a short window of cell cycle time cannot form rapidly in the absence of Cdc3-profilin. However, F-actin required in other processes (Novick and Botstein, 1985) might be assembled more slowly and/or might be formed by mechanisms not involving Cdc3-profilin.

Profilin overexpression experiments presented in this paper provide strong in vivo evidence that profilin can act as an actin-monomer sequestering protein. Cells overproducing Cdc3-profilin become elongated and dumbbell shaped, and the majority of these cells arrest at cytokinesis. Staining with rhodamine-conjugated phalloidin failed to detect any F-actin containing structures; the dots, patches, and the contractile ring found in wild-type cells were absent. The simplest interpretation of this observation is that profilin, when present above a certain level, loses some aspect of its regulation and acts as an actin-monomer sequestering protein. The experiments involving overproduction of both actin and Cdc3-profilin support that actin monomer sequestration is the probable cause of the phenotype observed in cells overproducing Cdc3-profilin; 60–70% of these cells are morphologically wild type. Staining with rhodamine-conjugated phalloidin revealed that F-actin was once again present in dots, patches, and rings in these cells. A similar but complementary set of experiments performed in the budding yeast had earlier pointed to the ability of profilins to sequester actin monomers in vivo (Magdolen et al., 1993). Overproduction of the prototypic actin Act1p in budding yeast cells results in lethality (Shortle et al., 1982). This lethality can be reversed by the overproduction of Pfy1p in cells overproducing Act1p (Magdolen et al., 1993). A role for profilin in sequestering actin monomers had also been proposed from studies in cultured human cells, where microinjection of profilin was shown to cause loss of F-actin structures (Cao et al., 1992).

We have identified the mutation in the Cdc3-124 mutant protein, since it represents the only available conditional mutation in any profilin studied thus far. This mutation results in substitution of a glutamic acid at position 43 by a lysine. The crystal structure of the *Acanthamoeba* profilin I isoform has been solved recently (Vinson et al., 1993). It has been shown that this protein, which is 48% identical in sequence to Cdc3-profilin, is composed of seven  $\beta$  sheets and three  $\alpha$  helices (Vinson et al., 1993). Assuming a similar structure for Cdc3-profilin, the mutation identified in the Cdc3-124-

profilin maps to the second  $\alpha$  helix. At present, the function of this region of profilin is unclear. Amino acid position 43 does not appear to be near the actin or phosphoinositide binding regions of profilin. One possibility is that this mutation alters the folding of the protein, rendering it inactive. This explanation seems reasonable since the phenotype of cells lacking Cdc3-profilin is very similar to the phenotype of *cdc3-124* cells under restrictive conditions. This explanation is also strengthened by the finding that overproduction of the E43K mutant profilin restores the threshold level of profilin activity required for cells to carry out cytokinesis, although these cells showed abnormalities at the level of F-actin structure. At present, we cannot explain the phenotype of cells overproducing the E43K mutant profilin. Biochemical characterization of the wild-type and E43K mutant will be necessary to understand the defect in the E43K mutant profilin.

In conclusion, this study has provided in vivo evidence for two diverse biochemical functions performed by profilins, actin filament formation and actin monomer sequestration. In addition, this study has also identified an amino acid residue essential for profilin function in vivo. With the availability of a conditionally lethal mutant in Cdc3-profilin it should be possible to identify genes whose products might interact with Cdc3-profilin. We have initiated genetic suppressor analyses and indeed have isolated several extragenic suppressors of the *cdc3-124* mutation. Further analyses of these suppressors is in progress. Analysis of profilin structure/function relationships should also be facilitated, since the inability to perform cytokinesis can be used as a criterion to isolate additional mutations in this gene. These studies, together with the wealth of biochemical studies and genetic studies in budding yeast and flies, should help us to understand more precisely the roles of profilins.

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