

Fission yeast Sop2p: a novel and evolutionarily conserved protein that interacts with Arp3p and modulates profilin function

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Profilins bind to monomeric actin and also interact with ligands such as phosphoinositide 4,5-bisphosphate, the proline-rich protein VASP and a complex of four to six polypeptides identified in *Acanthamoeba* that includes two actin-related proteins. Here, we report the identification and characterization of an essential gene from *Schizosaccharomyces pombe*, *sop2*⁺, a mutation in which rescues the temperature-sensitive lethality of a profilin mutation, *cdc3-124*. The *sop2-1* mutant is defective for cell elongation and septation, suggesting that it is involved in multiple cortical actin-requiring processes. Consistent with a role in actin cytoskeletal function, negative interactions have been identified between *sop2-1* and *act1-48*, a mutant allele of actin. Sop2p is a novel 377 amino acid polypeptide with similarity to proteins of the β -transducin repeat family. Sop2p-related proteins have been identified by sequencing projects in diverse species, and we have isolated a human cDNA highly related to *sop2*⁺, *SOP2 Hs*, which functionally complements the *sop2-1* mutation. Sop2p proteins from all species contain peptide sequences identical or highly similar to two peptide sequences from an *Acanthamoeba* β -transducin repeat protein present in the profilin binding complex. Biochemical analyses demonstrate that Sop2p is present in a complex which also contains the actin-related protein, Arp3p. Immunofluorescence studies reveal the presence of Sop2p in (i) punctate structures distributed throughout the cell, (ii) cables that extend the length of the cell, and (iii) a medial band in a small percentage of septating cells. Collectively these data demonstrate the interaction of Sop2p with Arp3p, profilin and actin.

Keywords: actin/actin-related protein/cytoskeleton/
profilin/*Schizosaccharomyces pombe*

Introduction

Profilins are small proteins identified in all eukaryotic cells in which they have been sought. Over the last 20 years, a wealth of biochemical information has been gathered which postulates the potential multifunctionality of this protein *in vivo*. *In vitro*, profilins have been found to interact with at least five ligands: (i) monomeric actin (Markey *et al.*, 1981); (ii) phosphoinositide 4,5-bisphosphate (Goldschmidt-Clermont *et al.*, 1991); (iii) poly-L-proline

(Tanaka and Shibata, 1985); (iv) the phosphorylated focal adhesion protein VASP (Reinhard *et al.*, 1995); and (v) a cortical complex comprising four to six polypeptides including the actin-related proteins Arp2 and Arp3 identified in *Acanthamoeba* (Machesky *et al.*, 1994; Kelleher *et al.*, 1995). From such biochemical studies, profilin has been proposed to either stimulate actin filament assembly in conjunction with thymosin- β 4 by lowering the critical concentration of actin or sequester actin monomers when the barbed ends of actin filaments are capped (Pantaloni and Carlier, 1993). Profilin has also been proposed to stimulate actin filament assembly by accelerating the rate of exchange of nucleotide bound to actin (Goldschmidt-Clermont *et al.*, 1992). Binding of profilin to PIP₂, polyproline-containing proteins and the cortical complex have been proposed to regulate the activity and/or localization of profilins (Machesky and Pollard, 1993).

An equally complex array of functions related to the actin cytoskeleton have been identified for profilins *in vivo* in experimental systems amenable to genetic and cytological manipulation. In *Dictyostelium*, *Tetrahymena* and the fission yeast *Schizosaccharomyces pombe*, profilins appear to function in cytokinesis (Edamatsu *et al.*, 1992; Balasubramanian *et al.*, 1994; Haugwitz *et al.*, 1994). In *Saccharomyces cerevisiae*, profilin is essential for growth at high temperatures, and its absence severely affects cell morphology at all temperatures (Haarer *et al.*, 1990). In *Physarum*, distinct isoforms are expressed in the plasmodial and amoeboid forms (Binette *et al.*, 1990). In *Drosophila*, profilin isoforms and profilin levels affect various developmental processes such as oogenesis, bristle morphogenesis, gastrulation and axonal growth (Cooley *et al.*, 1992; Veyerhen and Cooley, 1994).

In order to understand further the molecular function(s) of profilin, we chose to perform genetic suppressor analysis with the conditionally lethal *S.pombe* profilin mutant, *cdc3-124*. We reported earlier that the lesion in the mutant gene changed a glutamic acid residue present in the second α -helix (Vinson *et al.*, 1993; Federov *et al.*, 1994) (a region not thought to influence binding of profilin to actin, PIP₂ or poly-L-proline) to a lysine residue (Balasubramanian *et al.*, 1994). We used this mutant to isolate other genetic elements which reversed the temperature-sensitive lethality of *cdc3-124*. We report here the characterization of one extragenic suppressor, *sop2-1* (suppressor of profilin). *sop2-1* mutants are defective in cortical actin-dependent processes such as polarized cell elongation and septation. *sop2*⁺ is essential for cell viability and encodes a 41 kDa protein weakly related to proteins with β -transducin repeats. Sop2p is visualized in punctate structures distributed throughout the cell, cables that run the length of the cell and in the medial region of the cell during septation. Sop2p sediments in a multi-protein complex and is associated with the actin-related protein

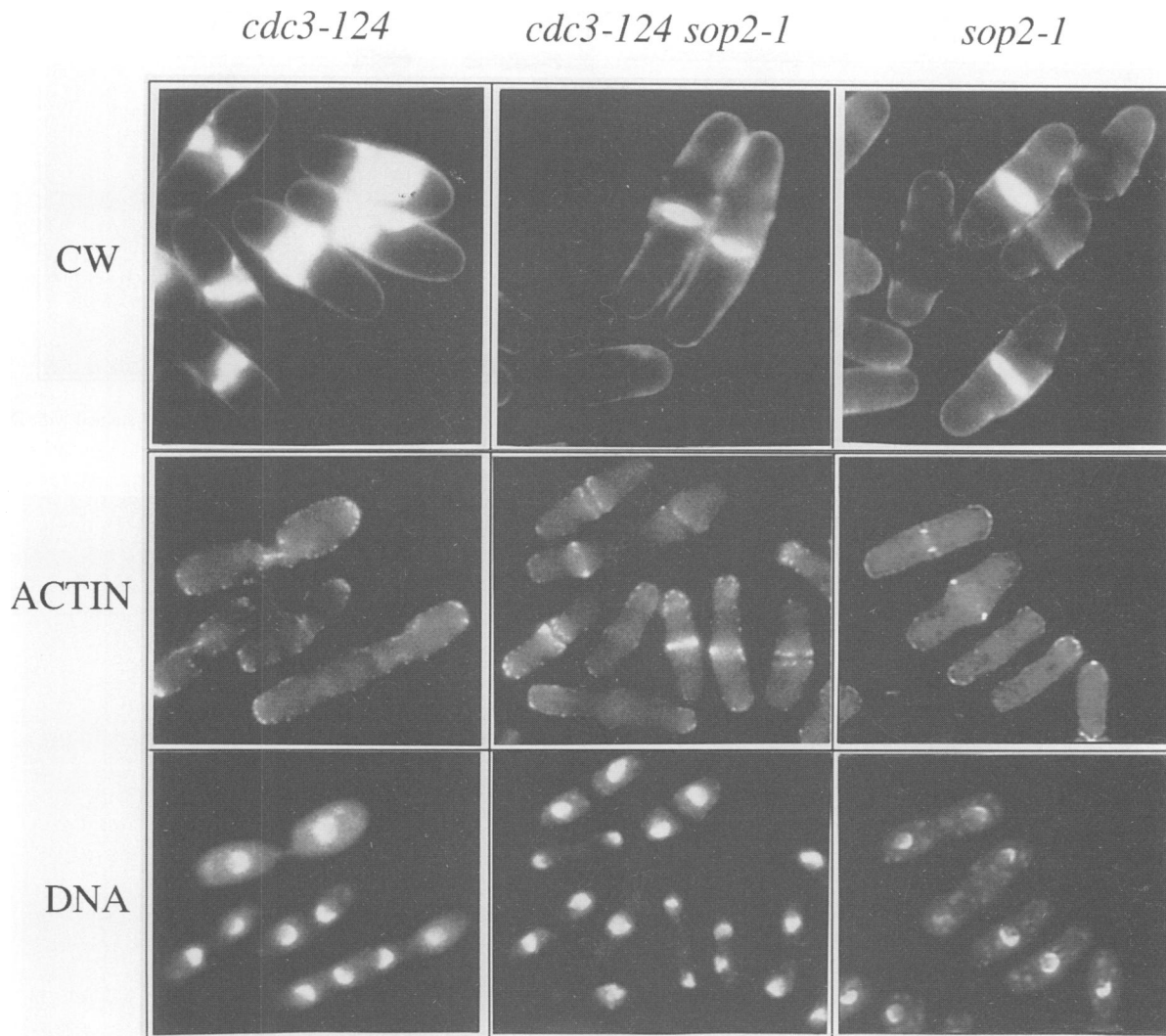


Fig. 1. Rescue of *cdc3-124* mutants by *sop2-1*. Cells of the indicated genotypes were grown at 29°C to log phase and shifted to 33.5°C for 4 h, fixed and stained with rhodamine-conjugated phalloidin (ACTIN) and DAPI (DNA), or calcofluor for cell wall (CW).

Arp3p. These data demonstrate the interaction of Sop2p with Arp3p, profilin and actin.

Results

Isolation and characterization of *sop2-1*

To identify genetic elements that might interact with the *cdc3-124* mutant profilin and render it functional (i.e. allow colony formation under restrictive temperature conditions), we performed a pseudoreversion screen. Although 36°C was used as the restrictive temperature for *cdc3-124* in previous studies (Nurse *et al.*, 1976; Balasubramanian *et al.*, 1994), we determined that *cdc3-124* cells fail to form colonies at 32°C or higher. We chose, therefore, to isolate pseudorevertants at 32°C to enhance our chances of identifying genetic interactions, under the assumption that even weaker interactions might be identified. From 2×10^8 mutagenized cells 1040 pseudorevertant colonies were isolated. We have studied 140 randomly chosen mutants in some detail and have determined that 119 of these suppression events were due to extragenic mutations while the other 21 were intragenic. Twenty of the 119 extragenic suppressors conferred cold-sensitive lethal

phenotypes at 19°C. The characterization of a linkage group consisting of a single allele, *sop2-1* (suppressor of profilin mutant), is the subject of this study.

Although higher temperatures did not affect growth of the *sop2-1* single mutant, the *sop2-1 cdc3-124* double mutant could not grow at temperatures above 33.5°C. Distribution of F-actin and cell wall material in *sop2-1*, *cdc3-124* and *cdc3-124 sop2-1* mutants at 33.5°C was examined by fluorescence microscopy (Figure 1). As described previously, *cdc3-124* mutants arrested at cytokinesis as binucleate or tetranucleate cells with improperly deposited septum material in the medial region of the cell (Balasubramanian *et al.*, 1994). F-actin contractile rings were not detected in these cells, although punctate F-actin staining was visualized. In contrast, both *cdc3-124 sop2-1* and *sop2-1* mutants formed contractile rings and septa at 33.5°C. However, some aspects of F-actin localization were affected in *sop2-1* and *cdc3-124 sop2-1* mutants. Whereas wild-type cells in mitosis display a prominent medial ring and minimal F-actin staining at the ends of the cells (data not shown), *sop2-1* and *cdc3-124 sop2-1* mutant cells with F-actin rings also contained copious punctate F-actin staining at the cell ends.

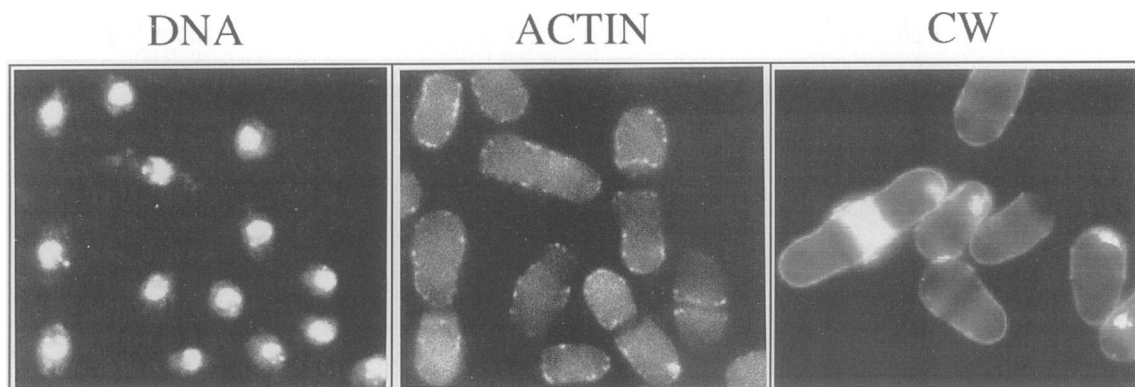


Fig. 2. Phenotype of *sop2-1* at 19°C. *sop2-1* cells were grown at 32°C to mid-log phase and shifted to 19°C for 10 h, fixed and stained with DAPI (DNA) and rhodamine-conjugated phalloidin (ACTIN) or calcofluor for cell wall (CW).

Approximately 45% of exponentially growing *sop2-1* mutant cells contained septa at 32°C. This was in marked contrast to the 10–12% of septating cells in wild-type cell cultures. We therefore entertained the possibility that a general slow down of septation and cell separation was the reason for rescue of *cdc3-124* by *sop2-1*. If this were the case, *sop2-1* should be capable of rescuing other mutants defective for actin ring formation/septation. To test this possibility, double mutants between *sop2-1* and two late septation mutants, *cdc8-110* and *cdc12-112*, and one early septation mutant, *cdc11-123*, were constructed and tested for colony formation at a variety of temperatures. This analysis showed that *sop2-1* was not capable of rescuing *cdc8-110*, *cdc12-112* and *cdc11-123* mutants.

Having established that *sop2-1* was specific in its ability to rescue *cdc3-124*, we characterized the phenotype of *sop2-1* at its restrictive temperature of 19°C. The *sop2-1* mutant was incapable of colony formation at 19°C and was sensitive to temperatures below 23°C. *sop2-1* was dominant, as determined by the inability of a *sop2-1/sop2+* diploid strain to form colonies at 19°C. The ability of *sop2-1* to suppress *cdc3-124* was not dominant, however, since a *cdc3-124/cdc3-124 sop2-1/sop2+* (O-16/MBY50) diploid could not form colonies at 33.5°C. Upon shift to 19°C, *sop2-1* cells arrested cell division with two major phenotypes before they eventually lysed. Between 50 and 60% of the cells contained a thick septum. In these cells F-actin was present in patches on both sides of the septum and all around the cell (Figure 2). The remainder of the arrested cells lacked a septum, and F-actin was present in delocalized patches. Occasionally, cells with multiple nuclei and multiple septa were identified. Calcofluor staining revealed that septum material was present in the cell ends in many cells that arrested in interphase. This is not observed in wild-type interphase cells. These phenotypes suggested that the *sop2+* gene product might be required for multiple processes that depend upon cortical actin, such as cell elongation and septation.

Fission yeast cells starved for a nitrogen source arrest in the G₁ phase of the cell cycle as small rounded cells. When a nitrogen source is added back to these cells, however, they re-enter the mitotic cell cycle, resume polarized growth and assume a cylindrical shape. We used this system to test specifically if *sop2+* was required for cell elongation. *sop2+* and *sop2-1* cells were arrested in the G₁ phase of the cell cycle by nitrogen starvation at

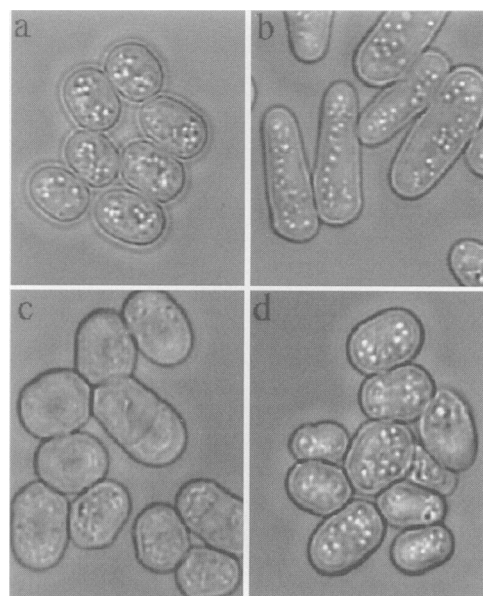


Fig. 3. *sop2+* is required for cell elongation. *sop2-1* cells were grown in minimal medium to mid-log phase at 32°C, washed three times with medium lacking a nitrogen source, inoculated into medium lacking a nitrogen source at 2×10^6 cells/ml and incubated at 32°C for 23 h followed by incubation at 19°C for 1 h. Cells were then inoculated ($t = 0$) in fresh minimal medium pre-cooled to 19°C at 2×10^6 cells/ml, incubated at 19°C for 16 h and then viewed under phase contrast settings. As a control, wild-type cells (972 h⁻¹) were treated in an identical manner. (a) Wild-type cells $t = 0$, (b) wild-type cells $t = 16$ h, (c) *sop2-1* cells $t = 0$ and (d) *sop2-1* cells $t = 16$ h.

32°C. This resulted in the accumulation of ~60% of *sop2-1* cells and >90% of wild-type cells as small rounded cells (Figure 3a and c). Flow cytometry confirmed the accumulation of both *sop2-1* and wild-type cells in G₁ (data not shown). Upon release into growth medium at 19°C, wild-type cells proceeded to elongate, assumed a cylindrical morphology and began to cycle (Figure 3b). In contrast, *sop2-1* mutants remained round and did not resume polarized growth (Figure 3d). Any growth that occurred under these conditions caused the cells only to swell. This result confirmed the requirement of *sop2+* for polarized cell elongation.

Molecular cloning and characterization

Although *sop2-1* was a dominant mutation, we reasoned that we might be able to clone *sop2+* by transformation

TAAAAATCTCTAAAAGATATGCAAGAAAATCAAATTTTATTTAAATGGAATTTTTTTTAG -203
 TCCTAATGAGTGTAAATTTTCAAAACCTTTAAAATGTAACAAGAGGATTACCGATAC -143
 CATCGATATGCGTTATCGTATATAAAGTACTCTAAACGTTTTTTGTTAATGTATCTACTGC -83
 TCTCGTCATCCAAATATCGATTCAGCTTCCGATATCGTCTATCATCCGCAAAAACAGTAG -23
 ACTGCTAGTCTGTTACTGAAAATGGCTTACCCTCAAGTTTACACATCTTCTCAAAC 37
 M A T S Q V L H I L P K 12

MluI
 CCTCTACGAACACCGCTTCAATAGTCAAAGAACGgtacagtataggacctaataaagt 97
 P S Y E H A F N S Q R T 24
 agattttaacaatttgatatagAGTTTGTCCACCACACTGCTACGAACCAAGTTGAAC 157
 E F V T T T A T N Q V E L 37
 TTACGAGCAAGATGGAAATGGGTGGAAGCATGCCCGAACATTTAGCGATCAGGATAAGAT 217
 Y E Q D G N G W K H A R T F S D H D K I 57
 TGTAACTTGTGCGATTGGGCTCCGAATCCAAATCGAATCGTAACTTGTCTCAAGACCG 277
 V T C V D W A P K S N R I V T C S Q D R 77
 TAATGCGTATGTTTATGAGAAACCTCTGATGGGACTTGAAGCAGACCCCTCGTCTACT 337
 N A Y V Y E K R P D G T W K Q T L V L L 97
 TCGTTGAATCGTCCGCTACGTTTGTTCGTTGGTCCGCAACGAGGACAAAGTTTGTCTGT 397
 R L N R A A T F V R W S P N E D K F A V 117
 TGGTAGTGTGCTCGTGTCAATTCCTGTCTACTTGTGAACAAGAAAATGACTGGGTGGT 457
 G S G A R V I S V C Y F E Q E N D W V 137
 CAGCAACATCTGAACCGTCCGCTCTGATGAGGACTTGAAGCAGACCCCTCGTCTACT 157
 S K H L K R P L R S T I L S L D W H P N 157
 TAACGTTTATAGTCCGCTGTGCGTGTGAGCAGCCTAAGCATACGTTTATCTGCTATGT 577
 N V L L A A G C A D R K A Y V L S A Y V 177
 TCGTAGTGTGATGCCAAACAGCAGGACCGCTTACCGTTACCTTTACCTTCAACAC 637
 R D V D A K P E A S V W G S R L P F N T 197

GAT
 TGTTCGCGTAATACCCATCTGTTGGGTTACGCGTGGATTTCACCTTCGGG 697
 V C A E Y P S G G W V H A V G F S P S G 217

D
 TAATGCGTATGCTAGTCCGCTATGCTTCTGTTACGATTGCATATCCAGTGTCC 757
 N A L A Y A G H D S S V T I A Y P S A P 237
 CGAGCAACCCGAGCTCTAATCTGTAACAAATTCCTCAATTCGCTTGGCTTCTCT 817
 E Q P P R A L I T V K L S Q L P L R S L 257
 TCTTTGGCGAATGAGAGTGCATTTGGCTGTGCTGCTTAACTATTCCTATCTTTTT 877
 L W A N E S A I V A A G Y N Y S P I L L 277

NruI
 GCAAGGCAATGAGTCCGGTGGGCCATCTACTCCGCAATTTGGATGCCGAACTTCCAAGAC 937
 Q G N E S G W A H T R D L D A G T S K T 297
 CTCATTCACTACACAGGAATACTGGTGAAGTAGAGGAGAGAGGGTCTGTTTCATT 997
 S F T H T G N T G E G R E E E G P V S F 317
 TACTGCTCTTGTGACACATTCGTAACATGGATTTGAAGGGCTCCAGCCAGTCTATTTC 1057
 T A L R S T F R N M D L K G S S Q S I 337
 GTCATTACCAACCGTTCATCAAAATGATGATGCAACCTTCGACCTTACGGTGGGATCC 1117
 S L P T V H Q N M I A T L R P Y A G T P 357
 TGGCAATATTACTCGGTTTACTCTCAGCGGTACCGAGCGGAGTGTGTTTGGTGGACTCT 1177
 G N I T A F T S S G T D G R V V L W T T 377
 TTAGAAAAGATATCGGCAATGAATCTTCAATCAATATTCGAATTTCCGATAGATT 1237

 AATCTCATAGATAAAGAATTTGAAACAGCTTTCAGCTAGATATAGTAACTGTTATTCGGC 1297
 ACGTAAATATGATCTATGTCCTTATAAATTTTGTCAATCATATTCAGTTTGTGT 1357
 ATATATTTTCTTACGAGACCCAAATTTGAGAAAGTCTTTGCTACGTAAGTTACTTA 1417
 TACCACCCTGAGACTGAAACCGGATTCGCTTCTACGAAATATCATAGATATAAC 1477
 TTAGATTAAAGTGTATTAATTTGATAGTTAGTAAAATTTAGTAAAGATC 1527

Fig. 4. Nucleotide sequence of *sop2*⁺ and predicted amino acid sequence of Sop2p. The nucleotide sequence has been deposited under the accession number Y08998. The predicted amino acid sequence is shown below the nucleotide sequence. Intron sequences are shown in lower case. The region of the gene between the *MluI* and *NruI* sites was deleted and replaced with the marker gene *ura4*⁺ to create the *sop2::ura4* gene disruption allele. The nucleotide mutated in the *sop2*-1 allele and the codon 207, which it affects, are shown. The amino acid change resulting from this mutation is shown below the wild-type amino acid sequence of Sop2p.

with a high copy plasmid library as elevated expression of *sop2*⁺ would dilute Sop2-1p and thereby rescue *sop2*-1. A *sop2*-1 *ura4*-D18 strain was therefore transformed with an *S.pombe* genomic library and two identical plasmids capable of rescuing *sop2*-1 were isolated. A DNA probe derived from the insert of these plasmids hybridized to cosmid 14C8c in a set of ordered cosmid clones representing the *S.pombe* genome (Hoheisel *et al.*, 1993). This localization placed the cloned gene between the *mat3* and *his2* loci. Subsequent genetic crosses revealed tight linkage between the *sop2* and *leu1* loci (<9 cM from *his2*) and also between *sop2* and *mat3* loci, suggesting that the cloned gene was *sop2*⁺ rather than a multicopy suppressor.

The DNA sequence of the 2.3 kb insert in the rescuing plasmid was determined (Figure 4). Analysis of this sequence revealed that it had the potential to encode a 377 amino acid polypeptide, from two putative exons separated by a 47 bp intron. The molecular weight for this predicted polypeptide is 41 638 Da. The existence and position of the predicted intron was confirmed by

isolation and sequence determination of *sop2*⁺ cDNA clones. Two findings established that this coding region was responsible for *sop2*-1 rescue. First, deletion of an internal segment encoding 271 amino acids abolished the ability of this plasmid to rescue *sop2*-1 mutants. Second, a single mutation which would change amino acid 207 from glycine to aspartic acid was identified when genomic DNA from the corresponding region in the *sop2*-1 mutant was sequenced (Figure 4).

The most recent protein databases were searched with Sop2p as the query sequence using the BLASTP program. These searches revealed that Sop2p was highly related to a predicted reading frame YBR234c (EMBL accession number Z36103) from *S.cerevisiae* and weakly related to several transducin repeat-containing proteins. The alignment of Sop2p and YBR234c indicated that they shared 40% identity, over the entire length of the two proteins, and 60% similarity when conservative amino acid substitutions were allowed. Subsequently, the DNA databases were translated in all potential frames and searched with Sop2p as the query sequence using the program TBLASTN. This search further identified a *Drosophila* genomic DNA sequence (DRO28DC9Z), several human expressed sequence tag (EST) sequences and a sequence from a mouse embryonal carcinoma cell line (MUS87F08) that were highly related (at least 60% identity within a stretch of 100 amino acids) to Sop2p. We found that the *Drosophila sop2*-like gene contained a potential intron at a position identical to that found in *sop2*⁺. We amplified a *sop2*-related human EST by PCR. The amplified sequence was used to probe a HeLa cell cDNA library and the nucleotide sequence of the full-length *sop2*-like gene from humans (referred to as *SOP2 Hs*) was determined. Figure 5A shows an alignment between Sop2p, *SOP2 Hs* and highly related sequences from *Drosophila*, mouse and *S.cerevisiae*. We have been able to identify five regions (amino acids 49–82, 114–138, 188–231, 254–282 and 359–377) which weakly resemble transducin repeats in Sop2p and its relatives.

Given the high degree of sequence similarity between Sop2p and related sequences from other species, we addressed if these proteins were functionally homologous. In other words, we asked if *SOP2 Hs* would rescue the *sop2*-1 mutant. The *SOP2 Hs* gene was placed downstream of the thiamine-repressible *nm1* promoter (Maundrell, 1989, 1993), introduced into the *sop2*-1 mutant and colonies were allowed to form at 32°C. As controls, an empty plasmid and a plasmid carrying the *S.pombe sop2*⁺ gene were also introduced into *sop2*-1 mutant cells. Colonies that formed at 32°C were replica plated to 19°C and scored for their ability to grow under these conditions. As shown in Figure 5B, *SOP2 Hs* and *S.pombe sop2*⁺, but not the empty vector, rescued the *sop2*-1 mutant. Thus, *SOP2 Hs* is a functional homolog of *S. pombe sop2*⁺.

Recently, Machesky *et al.* (1994) purified a complex of polypeptides which bound specifically to a profilin-agarose column and obtained peptide sequences from each of them. Three oligopeptide sequences from a protein, p40, were reported. This protein was identified as a putative β -transducin repeat protein based on one peptide sequence (amino acid sequence LVWDVASLVK). The other two peptide sequences from p40 were novel. We have found regions in Sop2p and Sop2p homologs from

A

<i>S. pombe</i>	1	M ATSQVLLHILPKPSPYEHAFNSQRTEFVTTATNQVELYEQDGNQWGHART	50
Human	1	M SLHQFLLEPITCCHAWNADRTQIALSPNNEHVHTYKKNQSGQWVAHE	47
<i>Dros.</i>	1	M AETFTFTGLASITCCHAWNDRDRTQIALSPNNEEIHHTYSREKSGDWKLLADV	50
<i>S. cere</i>	8	K SVVAVYKLVKAPITYSHCFSSQDKSILAVTCTDCLVYRVSNNTPEVLPAT	58
<i>S. pombe</i>	51	F SDHDKIVTCVDWAPKSNRIVTCSQDRNAYVYKRRDGTWKQTLVLLLRN	100
Human	48	L KEHNGHITGIDWAPKSDRIVTCSGADRNAYVWSQK.DGVWKPITLVLLLRN	96
<i>Dros.</i>	51	L NQHDLRVVGIDWAKNTRIVSCADRNAYVWTQGGDDGKWKPALVLLLRN	100
<i>S. cere</i>	59	L KDHDKTTITAVD.LTIEGRIVTCSQDRNAYVWEPLSDGTWKPTLVLLLRN	108
Mouse		<<<TWKPTLVLLLRN	
<i>S. pombe</i>	101	R AATFVVRWSPNEDKFAVGGGARVISVCYFEQENDWVSKHLKRPISRSTIL	150
Human	97	R AATFVVRWSPLENKFAVGGGARLISVCYFEENDWVSKHKKRPIRSTVL	146
<i>Dros.</i>	101	R AATCVRWSPLENKFAVGGGARLISVCYFEENDWVSKHKKRPIRSTVT	150
<i>S. cere</i>	109	R AATSVTWAPNGYKFAVGGGARIIAVCYFEENWVSKHKKRPIKSTIN	158
Mouse		R AARCVRWAPNENKFAVGGGARVISVCYFEQENDWVCKHKKRPIRSTVL	
<i>S. pombe</i>	151	S LDWHPNNVLLAAGCADRKAYVLSAYVRVDVAKPEASVWGSRLPFNTVCA	200
Human	147	S LDWHPNNVLLAAGSCDFKCRVFSAYIKKEVDEKASTFWGSKMFPGLQMS	196
<i>Dros.</i>	151	S LDWHPNNVLLAAGSDTYKRVVSAFIRKDEEPEPTTFWGNRKLPLGQLMA	200
<i>S. cere</i>	159	C LSWHANGVLLAAGGTDGFMRFVSGFLKGLDSESVAGSPWGQKFFFGCL	199
Mouse		S LDWHPNNVLLAAGSCDFKCRIFSAIYK>>>	
<i>S. pombe</i>	201	E YPS...GGWVHAVGFSPSGNALAYAGHDSVTTIAYPSAPEQFPRALI	245
Human	197	E FGGSGT...GGWVHGVRFSASGSLAWVSHDSTVSV...DASKSVQVS	240
<i>Dros.</i>	201	E FRNSQTSGGGWINSVFSDDGNKVCWVGHDSCVSIADATNGN...TVI	243
<i>S. cere</i>	247	I REWYQ...GSYIHDVWRSQMERIAYVAHDGTLNVVDYQSFPVQSVNA..	249
<i>S. pombe</i>	249	T VKLSQLFLRSLWANESAIVAAGYNYSPILLQGNESGWARTRDLDAQTS	295
Human	241	C LTFFVSKLDIP...KQSIQRNMSAMERFRNMDKRATTEDRNTALE	278
<i>Dros.</i>	247	R CRFGYLFPLSCEWVSPSTVAVAGYSCVPLLYSITADG.....	281
<i>S. cere</i>	253	P EGLPYRSLVWINDREIVCGGYSCHPVLFSASEGWKFAKMLDKSDN	299
<i>S. pombe</i>	296	K TSFTETG...NTGEGREEEGPVSFPTALRSTFRNMDLKGSSQSISSLPT	345
Human	279	C LTFFVSKLDIP...KQSIQRNMSAMERFRNMDKRATTEDRNTALE	320
<i>Dros.</i>	285	K LVLSGKL...DKSQKKESSGITAMRIFQSGMDRNMRTENTDTTVVDS..	341
<i>S. cere</i>	303	N KSALTASGNTDELSGNNDSSSTFGCISALRKFELDLKGV.STDVQES	353
<i>S. pombe</i>	346	V HQNMIIATLRFYAGTFGNITAFPTSSGTDGRVVLWTL*	377
Human	321	T LHQNSITQVSIYEVDEKQDCRKFCTTGIDGAMTIWDF.KTLESSIQGLRIM*370	
<i>Dros.</i>	342	I QHNAITSVRLYAGDKASATKVVSTSGVDGQLVIWVVEQGGINGGMRNLQI+377	
<i>S. cere</i>	354	A HENAIVELRPFASNSGQITQVSSCGLDGRVIYTTI*	384

C

KPTLVILR	Acanth	VFSAXIK	Acanth
KPTLVILR	Human	VFSAYIK	Human
KQTLVLLR	<i>S. pombe</i>	VLSAYVR	<i>S. pombe</i>
KPTLVLLR	<i>S. cerev</i>	VFSGFIK	<i>S. cerev</i>

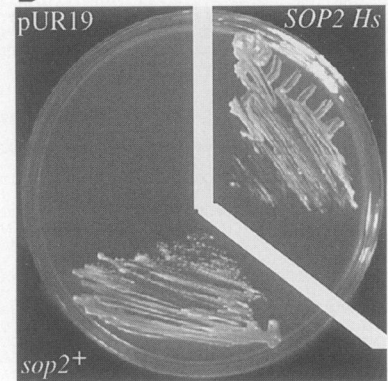
Fig. 5. (A) Alignment of the predicted amino acid sequences of Sop2p, *SOP2 Hs* and Sop2p-related sequences from *Drosophila*, mouse and *S.cerevisiae*. Sequences identical between Sop2p and at least one other member of this protein family are shown in bold letters. Gaps have been introduced to maximize alignment and are marked with periods. The nucleotide sequence of *SOP2 Hs* has been deposited under the accession number Y08999. (B) Rescue of *sop2-1* by *sop2+* and *SOP2 Hs*. Plate showing the ability of *S.pombe sop2+* and *SOP2 Hs*, but not an empty plasmid (pUR19), to rescue the *sop2-1* strain for colony formation at 19°C. (C) Alignment of peptide sequences identical or related to those present in *Acanthamoeba* p40, Sop2p, *SOP2 Hs* and the Sop2p-related gene in *S.cerevisiae*. Regions between amino acids 91 and 98 and 172 and 178 in Sop2p are shown. (D) Demonstration of *sop2*-deletion by genomic Southern blot analysis. DNA was prepared from the indicated strains, digested with *Hind*III and *Bgl*III, separated by agarose gel electrophoresis, blotted on to nylon membranes and probed with a ³²P-labeled probe encompassing the 1.6 kb *Bgl*III–*Hind*III fragment. The 1.6 kb band represents the *sop2+* locus, while the 2.5 kb band represents the *sop2::ura4* allele, in which the internal *Mlu*I–*Nru*I fragment has been deleted and replaced with the *ura4* gene. The *sop2::ura4* band is not as intense as the *sop2+* locus due to the deletion of ~800 bp of DNA with homology to the probe.

other species whose sequences are identical or highly related to the two novel peptide sequences from *Acanthamoeba* p40 (Figure 5C). The regions homologous to these two peptides are highly conserved among Sop2p family members and lie outside the β-transducin repeats. These analyses establish that Sop2p belongs to a highly conserved family of proteins and suggest that Sop2p might be functionally equivalent to *Acanthamoeba* p40.

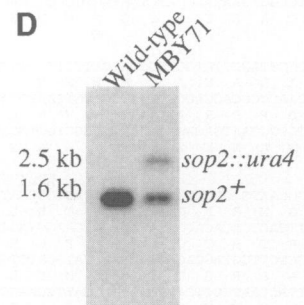
sop2 is essential for cell viability

Since *sop2* was identified on the basis of a dominant lethal mutation, it remained possible that the lethality in *sop2-1* cells was due to a gain-of-function of Sop2-1p. To study the phenotype of a *bona fide* *sop2* loss-of-function mutation, a diploid strain with the genotype *sop2::ura4/sop2+* (MBY71) was constructed as described in Materials and methods. Precise replacement of one of the copies of

B



D



sop2+ by *sop2::ura4* was confirmed by Southern blot analysis of genomic DNA prepared from MBY71 (Figure 5D). MBY71 was sporulated and tetrads were dissected. All dissected tetrads produced two Ura⁻ colonies while the other two spores lysed after germination. The lethality of the Ura⁺ spores was reversed if a plasmid carrying the *sop2+* cDNA was introduced into the heterozygous diploid prior to sporulation. Thus, *sop2+* is essential for cell viability.

Since *sop2::ura4* mutant spores lysed after germination, we were able to characterize the phenotype of *sop2::ura4* mutant cells only by the plasmid loss procedure. To this end, the *sop2+* cDNA under control of the *nmt1* promoter was introduced into the heterozygous diploid carrying the *sop2* null allele. The cells were sporulated, and haploid *sop2::ura4* cells rescued by a plasmid-borne *sop2* cDNA were isolated. Rescue was observed even when

transcription from the *nmt1* promoter was repressed, indicating that basal levels of transcription from the *nmt1* promoter produced sufficient amounts of Sop2p to rescue *sop2* null mutants. Plasmid-rescued *sop2::ura4* cells were grown in medium containing leucine. Passage through the mitotic cell cycle allows ~10% of cells to lose the autonomously replicating plasmid. Due to the presence of leucine in the medium, cells which had lost the plasmid were capable of metabolic growth and arrested the cell cycle with phenotypes indicative of the functional execution point(s) of Sop2p. The phenotype of the *sop2* null mutant was very similar to that of the *sop2-1* mutant. In particular, cells with thick septa and punctate actin distributed all around the cell were identified, as well as uninucleate cells with punctate F-actin structures distributed around the cell (data not shown). Thus, the cold-sensitive *sop2-1* mutant appears to be a dominant-negative allele of *sop2*.

Sop2p is present in a high molecular weight complex

To characterize the *sop2*⁺ gene product, antibodies were raised against a GST–Sop2p fusion protein expressed in *Escherichia coli* and affinity purified. This antiserum recognized a single band of M_r 45 kDa in *S.pombe* total cell lysates by immunoblotting (Figure 6A). In total cell lysates prepared from cells overexpressing the *sop2*⁺ cDNA, the band detected by immunoblotting increased in intensity (Figure 6A). As described earlier, Sop2p contains sequences related to those present in the 40 kDa WD40/transducin repeat protein present in the high molecular weight cortical complex that interacts with profilin in *Acanthamoeba*. To determine whether Sop2p behaved similarly in *S.pombe*, we asked the following questions. (i) Is Sop2p present in high molecular weight complexes in the cell? and (ii) does Sop2p bind Cdc3–profilin?

To assess the native size of Sop2p-containing complexes in *S.pombe* cells, cell lysates were prepared in a buffer known to solubilize components of the actomyosin cytoskeleton and loaded on to a 5–20% glycerol gradient. Fractions were collected after centrifugation and analyzed by Western blotting using affinity-purified antibodies to Sop2p and Cdc3p (Figure 6B). Sop2p was detected solely in a large molecular weight complex which migrated between myosin heavy chain (200 kDa) and β-galactosidase tetramers (464 kDa). Cdc3p, however, did not co-sediment with Sop2p and was detected towards the top of the gradient, suggesting that it existed predominantly as a monomer or in a complex with G-actin *in vivo*. The profilin binding complex in *Acanthamoeba* includes two actin-related proteins, Arp2p and Arp3p (Machesky *et al.*, 1994). Since *S.pombe* Arp3p was also detected solely in a large molecular weight complex (McCollum *et al.*, 1996), we asked if Sop2p and Arp3p co-migrated under density gradient centrifugation conditions, by immunoblotting fractions with Arp3p antibodies. As shown in Figure 6B, Arp3p co-migrated with Sop2p, suggesting that these two proteins may be complexed in cells.

The density gradient sedimentation of Sop2p and Cdc3p suggested that these two proteins are not stably associated *in vivo*. This was not surprising if Sop2p behaved similarly to *Acanthamoeba* p40, since profilin did not co-purify with the profilin binding complex although the profilin

binding complex bound specifically to profilin–Sepharose and PLP–Sepharose matrices (Machesky *et al.*, 1994). To address whether Sop2p bound Cdc3p, we asked if Sop2p could be co-purified with Cdc3p. We took advantage of the fact that the profilin binding complex co-purified with profilin following poly-L-proline affinity chromatography in *Acanthamoeba* lysates (Machesky *et al.*, 1994). *Schizosaccharomyces pombe* lysates were prepared and fractionated by high speed centrifugation and ion-exchange chromatography (since we had determined previously that the majority of profilin and Sop2p were soluble and were not retained in the DEAE-cellulose matrix). The flow-through from ion-exchange chromatography was loaded on a PLP–Sepharose column, washed extensively and eluted with increasing concentrations of urea. As shown in Figure 6C, a 13 kDa polypeptide bound tightly to PLP–Sepharose. A small fraction of this polypeptide eluted in the 4 M urea washes, while the vast majority eluted in the 6 M urea washes. Immunoblotting confirmed that this polypeptide was Cdc3p (marked with an arrow in Figure 6C). In addition, a 19 kDa polypeptide and a 120 kDa polypeptide bound and eluted when washed with 4 and 6 M urea respectively. The identity of the 19 and 120 kDa proteins is unknown. We failed to detect polypeptides of the size predicted for Sop2p among eluates from the PLP–Sepharose column by either Coomassie staining or immunoblotting, and Sop2p was detected in the flow-through from the PLP–Sepharose column (data not shown). Furthermore, we were unable to detect binding of Sop2p to a Cdc3p–Sepharose column. Immunoblotting using antibodies raised against the product of the *S.pombe* *arp3* gene (McCollum *et al.*, 1996) failed to reveal the presence of Arp3p in eluates of the profilin–Sepharose and PLP–Sepharose matrices (data not shown).

Although we could not detect binding between Sop2p and *S.pombe* Cdc3p, we asked if Sop2p was functionally related to the *Acanthamoeba* p40 by a different criterion. Given that the *Acanthamoeba* p40 was present in a high molecular weight complex which included the *Acanthamoeba* homolog of Arp3p, and that Sop2p and Arp3p co-sedimented by density gradient centrifugation analysis, we addressed whether Sop2p and Arp3p were contained within the same complex. Wild-type cells were metabolically labeled with ³⁵S (Trans label) and immunoprecipitations were carried out with antibodies against Sop2p or Arp3p. These primary immunoprecipitates were boiled in the presence of SDS to release constituents of the immune complex, and the supernatants were divided into two parts. One was immunoprecipitated with antibodies against Arp3p and the other was immunoprecipitated with pre-immune antibodies. As expected, Arp3p was present in primary immune complexes generated with antibodies against Arp3p (Figure 6D). Interestingly, Arp3p was also present in the primary immune complexes generated with antibodies against Sop2p. It was not feasible to detect Sop2p in Arp3p immunoprecipitates as Sop2p did not label well while Arp3p labeled very well, and also due to the fact that the Sop2p antibodies were inefficient in precipitating denatured Sop2p. Immunoblotting was also not practical since Sop2p and Arp3p nearly co-migrate with the heavy chains of IgG.

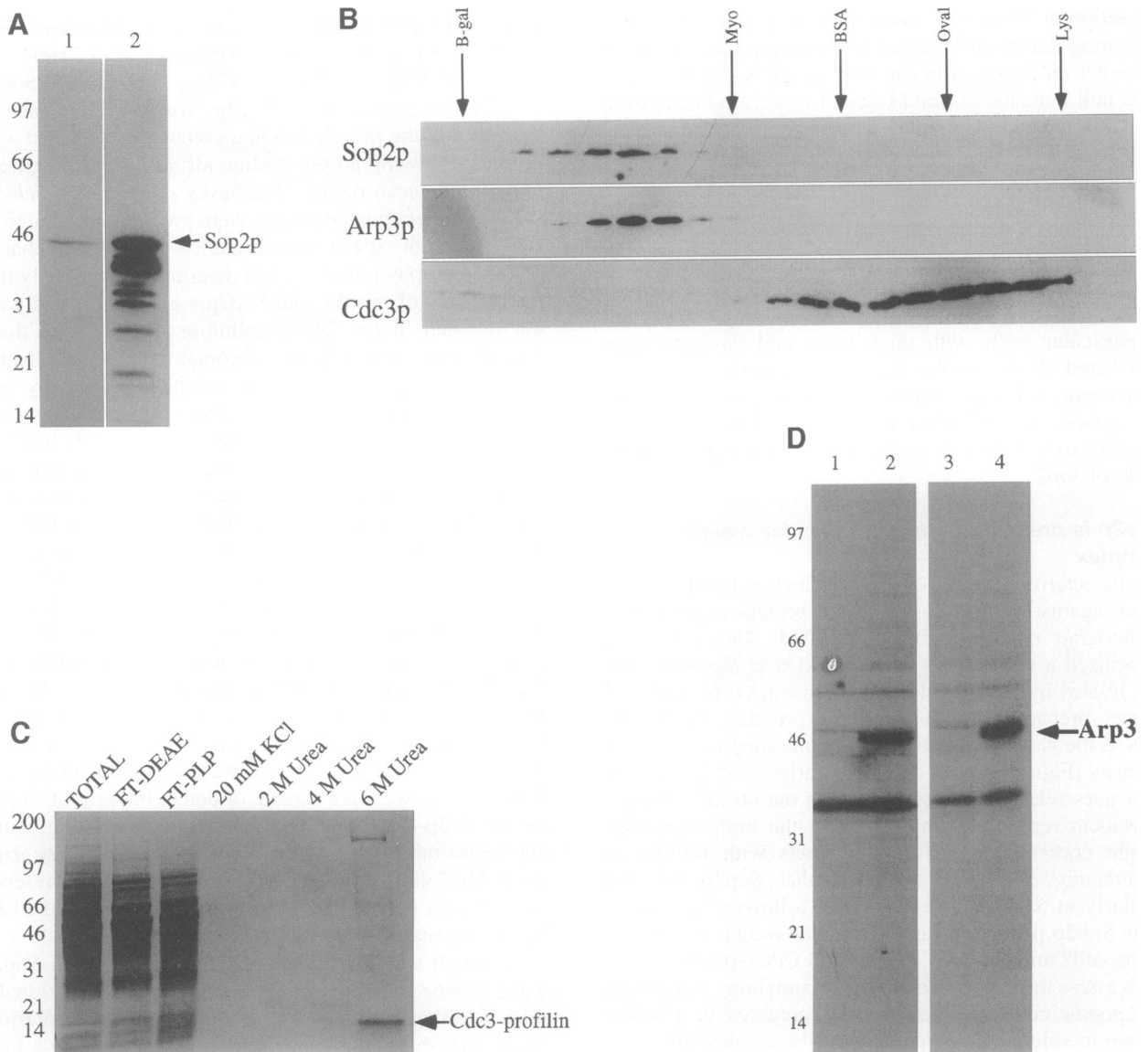


Fig. 6. Biochemical analysis of Sop2p, Cdc3-profilin and Arp3p. **(A)** Immunoblot of total proteins from wild-type (lane 1) and cells overproducing Sop2p (lane 2) with affinity-purified anti-Sop2p serum. **(B)** Density gradient sedimentation analysis of Sop2p, Arp3p and Cdc3p. Cell lysates were prepared and separated by density gradient centrifugation. Fractions from the gradient were analyzed by immunoblotting with antibodies against Sop2p, Arp3p and Cdc3p. Molecular size standards which were run in parallel and their positions in the gradient are shown above the immunoblot, and are as follows β -gal = 464 kDa, Myo = 200 kDa, BSA = 69 kDa, Oval = 45 kDa, Lys = 14 kDa. **(C)** Purification of Cdc3-profilin by PLP-Sepharose chromatography. Cell lysates were prepared and fractionated by high speed centrifugation and ion-exchange chromatography. The flow-through from this step was applied to a PLP-Sepharose column, washed and eluted as indicated in Materials and methods. Fractions were separated on a 4–20% gradient polyacrylamide gel and stained with Coomassie blue. **(D)** Sop2p and Arp3p are present in a complex in *S.pombe* cells. Wild-type cells were metabolically labeled with ^{35}S Trans label. Lysates were prepared from these cells and immunoprecipitated with antibodies against Arp3p (lanes 1 and 2) or Sop2p (lanes 3 and 4) under non-denaturing conditions to generate primary immune complexes. These primary complexes were boiled in SDS lysis buffer and immunoprecipitated with antibodies against Arp3p. The first two lanes represent primary complexes generated with antibodies against Arp3p, denatured and immunoprecipitated with pre-immune (lane 1) or immune Arp3p (lane 2) antibodies. The third and fourth lanes represent primary complexes generated with antibodies against Sop2p, denatured and immunoprecipitated with pre-immune (lane 3) or immune Arp3p (lane 4) antibodies.

Intracellular localization of Sop2p

Asynchronously growing wild-type cells were stained with anti-Sop2p antibodies to visualize the intracellular distribution of Sop2p (Figure 7). Pre-immune serum produced only background levels of staining (Figure 7), whereas cells stained with anti-Sop2p serum displayed several distinct staining patterns. First, Sop2p was excluded from the nucleus and localized in the cytoplasm (Figure 7). Second, Sop2p was localized to patches of varying sizes distributed throughout the cell and these

patches were not particularly enriched in the growing end(s) of the cells. Thirdly, Sop2p was present in cables that ran the length of the cells. DAPI staining of mitochondrial DNA suggested that, in many cases, mitochondrial DNA was placed along these cables. Finally, Sop2p was detected near the septum in a small fraction of septating cells.

The detection of Sop2p in cables was intriguing since cytoplasmic microtubules in *S.pombe* cells appear as cables that run the length of interphase cells (Hagan and

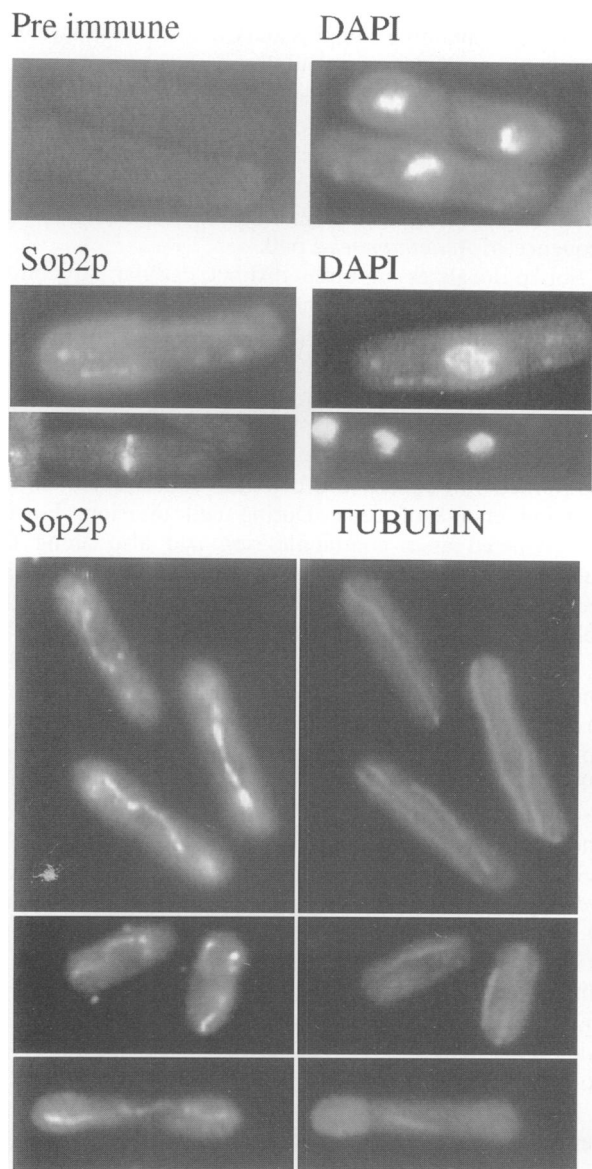


Fig. 7. Immunolocalization of Sop2p. Wild-type cells were fixed with a mixture of formaldehyde and glutaraldehyde or methanol and stained with antibodies against Sop2p or a mixture of antibodies against tubulin and Sop2p (Figure 7). In many interphase cells with distinct cytoplasmic microtubules, Sop2p was visualized in cables that ran the length of cells. These cables were not superimposable with cytoplasmic microtubules, suggesting that Sop2p was not present on cytoplasmic microtubules. However, since both microtubules and Sop2p cables run the length of the cylindrically shaped cells, the conclusion that Sop2p did not co-localize with microtubules remained ambiguous. Therefore, to establish clearly that Sop2p was not present on cytoplasmic microtubules, we asked if mitotic cells which are devoid of detectable cytoplasmic microtubules displayed Sop2p cable staining. Sop2p cables were visualized in mitotic

Hyams, 1988). To address if Sop2p co-localized with cytoplasmic microtubules in wild-type cells, double labeling was performed with antibodies against tubulin and Sop2p (Figure 7). In many interphase cells with distinct cytoplasmic microtubules, Sop2p was visualized in cables that ran the length of cells. These cables were not superimposable with cytoplasmic microtubules, suggesting that Sop2p was not present on cytoplasmic microtubules. However, since both microtubules and Sop2p cables run the length of the cylindrically shaped cells, the conclusion that Sop2p did not co-localize with microtubules remained ambiguous. Therefore, to establish clearly that Sop2p was not present on cytoplasmic microtubules, we asked if mitotic cells which are devoid of detectable cytoplasmic microtubules displayed Sop2p cable staining. Sop2p cables were visualized in mitotic

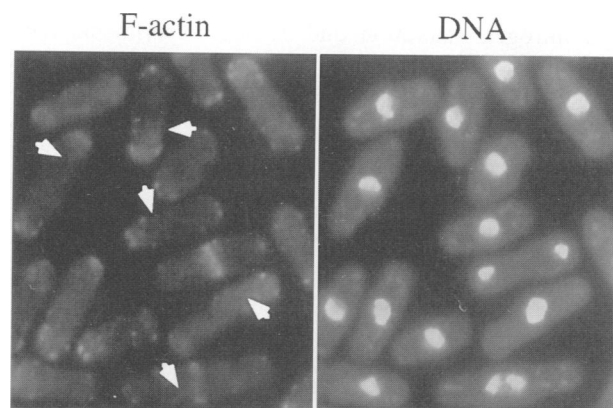


Fig. 8. Actin cables in fission yeast. Cells were stained with DAPI for DNA and with rhodamine-conjugated phalloidin for F-actin. Some actin cables have been marked with an arrow.

cells displaying only a mitotic spindle and lacking cytoplasmic microtubules, establishing that Sop2p did not co-localize with microtubules.

***sop2-1* and *act1-48* mutations display strong negative interactions**

In *S.pombe*, occasionally F-actin has also been visualized in cables that run the length of the cell (Marks and Hyams, 1985). A protocol that allows more reliable detection of F-actin cables was developed. Wild-type cells stained using this protocol showed F-actin in cables in addition to the patches and contractile ring structures that are usually visualized (Figure 8). Thus, it appears that F-actin is also present in cables that run from one end to the other of *S.pombe* cells. Given that Sop2p and F-actin were detected in cables, and that Sop2p did not co-localize with microtubules, it seemed probable that Sop2p was present on actin cables. We therefore assessed potential genetic interactions between *sop2-1* and the cold-sensitive actin mutant *act1-48* (D.McCollum and K.L.Gould, unpublished observations). *sop2-1* and *act1-48* were mated and tetrads were dissected. From this cross, colonies were recovered with the genotypes *sop2-1*, *act1-48* and *sop2⁺act1⁺* (wild-type) at 32°C. However, no *sop2-1 act1-48* double mutant colonies were recovered under conditions permissive for both single mutants. Microscopic examination revealed that the presumed double mutants lysed after germination. Thus *sop2-1* and *act1-48* mutations display synthetic lethality.

Discussion

A novel protein, Sop2p, has been identified through classical genetic suppressor analysis of a mutation in the *S.pombe cdc3* gene. The cold-sensitive mutant *sop2-1* rescues *cdc3-124* at a lowered restrictive temperature of 33.5°C, but not at 36°C. This suggests that the *sop2-1* is not a by-pass suppressor of *cdc3-124*. Rather, *sop2-1* might stimulate residual activity of the Cdc3-124p, or work by other mechanisms, as discussed below, to reverse the temperature-sensitive lethality of *cdc3-124*.

Sop2p is essential for cell viability. *sop2-1* and *sop2*-null mutants display delocalized actin patches and cell wall material, suggesting a role for Sop2p in organization of actin patches and in polarized growth. Consistent with

this, nitrogen starvation and release experiments have shown that *sop2-1* mutant cells are defective for cell elongation. Actin ring formation and contraction are not affected in these mutants. We have observed recently that a cold-sensitive mutation in the actin-related protein Arp3p, which is present in a complex with Sop2p, results in a phenotype similar to that observed for *sop2-1* and is capable of rescuing *cdc3-124* mutants (McCullum *et al.*, 1996). Interestingly, both *sop2-1* (this study) and *arp3-c1* (McCullum *et al.*, 1996) mutants display strong negative interactions with an actin mutant, *act1-48*. Thus, mutants which are phenocopies of *sop2-1* might represent a novel set of gene functions which modulate cortical actin functions *in vivo*. Mutants with these phenotypes have not been characterized previously in fission yeast.

The *sop2*⁺ gene encodes a 377 amino acid protein which is weakly related to proteins of the β -transducin repeat family. We have also reported the sequence of a human homolog of Sop2p, *SOP2 Hs*, expression of which rescues the cold-sensitive lethality of a *sop2-1* mutant. Other sequences highly related to Sop2p have been identified from genome sequencing projects. These studies taken together suggest that Sop2p is a member of a new family of proteins related to the β -transducin repeat family. Several proteins with a β -transducin repeat are known to function in multi-protein complexes and the β -transducin-repeat motif has been speculated to be involved in protein-protein interactions (Neer *et al.*, 1994; Neer and Smith, 1996). Consistent with this notion, we have observed that Sop2p is a part of a large molecular weight complex and is complexed with the actin-related protein Arp3p *in vivo*. In light of the fact that the β -transducin repeat motif has been proposed to be involved in protein-protein interactions, it is also interesting that the mutation identified in *sop2-1* lies within a β -transducin repeat (it falls within the β -transducin repeat 188–231, at position 207) raising the possibility that the Sop2p-containing multi-protein complex might be functionally compromised in the mutant.

At present, the molecular function of the Sop2p-containing complex is not known. The identification of a profilin binding complex in *Acanthamoeba* has directed our studies in this regard (Machesky *et al.*, 1994). This protein complex, which specifically bound a profilin-agarose matrix, contained the actin-related proteins Arp2 and Arp3, a 40 kDa protein with β -transducin repeats and a 35 kDa protein. Several of our results suggest that Sop2p is a functional homolog of the p40 β -transducin repeat protein identified in *Acanthamoeba*. First, Sop2p, *SOP2 Hs*, *SOP2 Dm* and *S.cerevisiae* Sop2p are approximately the same size as p40 and carry β -transducin repeating units. Second, Sop2p and its relatives from human, mouse, rat, *Drosophila* and budding yeast contain sequences identical or related to peptide sequences present in *Acanthamoeba* p40. Third, Sop2p is present in a complex containing the actin-related protein Arp3p. Finally, *sop2*⁺ interacts genetically with the profilin gene *cdc3*⁺, and a mutation in *sop2* was indeed isolated as a suppressor of the temperature-sensitive lethality of the *cdc3-124* mutant. In contrast to the results of Machesky *et al.* (1994), however, we have been unable to detect binding between the Sop2p/Arp3p complex and Cdc3-profilin. Possible explanations for our inability to detect binding between

the Sop2p-containing complex and Cdc3p include a weaker interaction between this complex and Cdc3p in fission yeast. Alternatively, some factor necessary for binding of the Sop2p-containing complex in *S.pombe* is labile under our purification conditions. Positive confirmation of whether Sop2p is the homolog of *Acanthamoeba* p40 will have to await the availability of the full-length amino acid sequence of *Acanthamoeba* p40.

Sop2p localizes in three distinct cellular structures: patches of varying sizes, cables and a medial band in a small percentage of septating cells. The pattern of Sop2p localization is not identical to that of any other known *S.pombe* protein. However, there are many similarities between the observed Sop2p staining and the localization of F-actin. During interphase, F-actin is present in patches distributed throughout the cell but concentrated in the growing end(s) of cells. During cell division, F-actin is visualized as a contractile ring and also along the subsequently formed septum. F-actin has also been visualized previously as thin cables that run the length of the cell (Marks and Hyams, 1985).

Given that Sop2p does not co-localize with microtubules and the similar patterns of Sop2p and F-actin staining, as in the case of the *Acanthamoeba* profilin binding complex co-localizing with the actin-rich cortex (Machesky *et al.*, 1994; Kelleher *et al.*, 1995), it is likely that Sop2p localizes to a subset of actin patches and to actin cables. Arp3p and Sop2p are present in the same complex in *S.pombe* cells and neither are part of the contractile ring. In contrast, we have not detected Arp3p in actin cables (McCullum *et al.*, 1996). The reason for this discrepancy is unclear. It is possible that Sop2p or Arp3p may not be accessible to antibodies at some locations or that the fixation procedures might have different effects on each of these antigens. Alternatively, it is possible that Sop2p and Arp3p do not co-localize at all times.

It is paradoxical that the *sop2-1* mutant, which is not defective in actin ring formation or contraction, is capable of rescuing the *cdc3-124* mutant, which is defective specifically for actin ring formation and cytokinesis. One possible explanation for the ability of *sop2-1* to rescue *cdc3-124* is that the Sop2p/Arp3p-containing complex might mediate a subset of profilin functions which are not required for profilin-mediated actin ring formation. Rather, the Sop2p/Arp3p-containing multi-protein complex might participate in profilin- and actin-requiring functions such as cell elongation, secretion and septation. Thus, in a *sop2-1 cdc3-124* mutant the Sop2p/Arp3p-containing multi-protein complex might interact weakly with Cdc3-124p and allow more of the Cdc3-124p to function in formation of the contractile ring. This model is supported by the following of our previous findings: (i) Cdc3-profilin localizes to the ends in growing cells and localizes to the medial region in cells undergoing cytokinesis, suggesting that it functions in both cell elongation and cytokinesis, and (ii) overproduction of Cdc3-124p rescues *cdc3-124* at 36°C (Balasubramanian *et al.*, 1994). Furthermore, we have isolated four alleles of a second gene, *sop1*, which also rescue *cdc3-124* mutants. Interestingly, *sop1* single mutants themselves are defective for cytokinesis, raising the possibility that Sop1p functions with Cdc3p specifically in actin ring formation

Table I. *Schizosaccharomyces pombe* strains used

Strain	Genotype	Reference
MBY15	<i>cdc3-124 ade6-M210 ura4-D18 h⁻</i>	this laboratory
MBY34	<i>ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h⁺/h⁻</i>	this laboratory
MBY50	<i>cdc3-124 ade6-M210 ura4-D18 leu1-32 h⁺</i>	this laboratory
MBY62	<i>sop2-1 ade6-M210 ura4-D18 h⁻</i>	this study
MBY71	<i>sop2::ura4/sop2⁺</i> MBY34	this study
MBY72	<i>sop2-1 h⁻</i>	this study
MBY73	<i>sop2-1 ade6-M210 ura4-D18 leu1-32 h⁻</i>	this study
MBY106	<i>cdc11-123 ade6-210 h⁺</i>	this study
O-16	<i>cdc3-124 sop2-1 ade6-M210 ura4-D18 h⁻</i>	this study
KGY249	<i>ade6-M216 ura4-D18 leu1-32 h⁺</i>	this laboratory
KGY281	<i>mei1-102 lys1-131</i>	this laboratory
KGY657	<i>cdc8-110 ura4-D18 leu1-32 h⁺</i>	this laboratory
KGY658	<i>cdc12-112 leu1-32 h⁺</i>	this laboratory
KGY1011	<i>act1-48 ura4-D18 leu1-32 lys1-131 h⁺</i>	this laboratory

(M.K.Balasubramanian and K.L.Gould, unpublished observations).

Further biochemical analysis will be required to understand the molecular function(s) of the Sop2p/Arp3p-containing complex in actin cytoskeletal regulation. The identification by genetics (McCollum *et al.*, 1996; this study) and biochemistry (Machesky *et al.*, 1994) of a multi-protein complex containing actin-related proteins Arp2p and Arp3p, and a protein with β -transducin repeats, Sop2p, suggests a fundamental role for this complex in cytoskeletal regulation. These roles can now be addressed for Sop2p and its human homolog with the reagents generated from this work.

Materials and methods

Strains, media and genetic methods

Schizosaccharomyces pombe strains used in this study are listed in Table I. Media used for vegetative growth (YEA and EMM2) and sporulation (ME) are described in Moreno *et al.* (1991). Cultivation of cells and genetic analysis were performed as described (Moreno *et al.*, 1991). Transformations were performed by electroporation (Prentice, 1991).

A total of 2×10^8 cells of a *cdc3-124 ade6-M210 ura4-D18 h⁻* strain were mutagenized with nitrosoguanidine and plated at the restrictive temperature of 32°C. This screen yielded 1040 colonies. One hundred and forty of these were mated individually to a wild-type strain and the products of meiosis were analyzed by the free spore method. Briefly, spores were plated at 25°C and allowed to form colonies which subsequently were replica plated to 36°C. The presence of Cdc⁻ progeny at 36°C was taken to imply extragenic suppression while the rest were classified as intragenic mutants. Mutation in one of the loci thus identified (*sop2-1*) resulted in cold-sensitive lethality. Dominance/recessivity tests were carried out using the following strains: *sop2-1 ade6-M210 ura4-D18 h⁻/sop2⁺ ade6⁺ ura4⁺ lys1-131 mei1-102* (MBY62/KGY281) and *sop2-1 ade6-M210 ura4-D18 h⁻/ade6-M216 ura4-D18 leu1-32 h⁺* (MBY62/KGY249). The use of a diploid strain without inter-allelic *ade6* complementation (*ade6-M210/ade6-M216*) was necessary due to the fact that an *ade6-M210/ade6-M216* diploid was partially cold-sensitive.

Cloning of *sop2⁺*, *sop2-1* and *SOP2 HS*

A *sop2-1 ade6-M210 ura4-D18 h⁻* (MBY62) strain was transformed to uracil prototrophy with an *S.pombe* genomic library constructed in the plasmid pUR19 (Barbet *et al.*, 1992). Subsequently, transformants were scored for colony formation at 19°C. From two independent transformations two colonies were identified at 19°C. Plasmids were recovered from these strains into the *E.coli* strain MC1061. Restriction mapping of the isolated plasmids revealed that they were identical and that they carried 2.3 kb of *S.pombe* DNA. The *sop2⁺* gene sequence was determined by sequencing from ends of subclones with the M13 universal and M13 reverse primers. Regions which were not accessible from these two primers were sequenced with synthetic oligonucleotide primers which were placed internally. Sequencing was performed at the

Vanderbilt Cancer Center using an ABI automated sequencer. The *sop2-1* mutant gene was isolated by PCR and sequenced by the dideoxy method. The *sop2⁺* cDNA was isolated by PCR from an *S.pombe* cDNA library constructed in the plasmid vector pDB20 (Fikes *et al.*, 1990). Sequence comparisons were carried out using the BLAST series of programs. A fragment of a human cDNA very highly related to Sop2p was identified as an EST from the EMBL database (Z44484). A DNA fragment internal to EST Z44484 was amplified by PCR and its sequence verified. The PCR fragment was then used to screen a HeLa cell cDNA library in the vector pcD (Okayama and Berg, 1981). The nucleotide sequence of the human gene was determined using synthetic oligonucleotide primers at the Vanderbilt Cancer Center DNA sequencing facility.

Deletion of the *sop2* gene

Plasmid MB110 was created by deleting the *MluI-NruI* fragment of the *sop2* gene, which encodes amino acids 20–288, and replacing it with a 1.8 kb fragment carrying the *ura4⁺* gene. This plasmid was linearized with *XhoI* and *XbaI* and the *XhoI-XbaI* fragment was transformed into the diploid strain MBY34. Ura⁺ transformants were replica plated five times at 1 day intervals to media containing uracil to allow loss of any autonomously replicating DNA molecules carrying the *ura4⁺* gene. Cells subsequently were replica plated to medium lacking uracil, and putative integrants were identified as clones that were still capable of colony formation under these conditions. Southern blot and PCR analysis established that the desired gene replacement event had taken place.

Generation of anti-Sop2p antibodies

The full-length *sop2⁺* cDNA was cloned into the *SmaI* site of plasmid GEX2T (Smith and Johnson, 1988) to allow production of a GST–Sop2p fusion protein. GST–Sop2p was soluble in aqueous solutions and was purified on a glutathione–agarose column. The soluble protein was used as an antigen to raise polyclonal antisera in two rabbits, VU32 and VU33. Antibodies were affinity purified as described in Olmsted (1981).

Density gradient analysis

Cell extracts were prepared in pyrophosphate buffer [150 mM KCl, 12 mM sodium pyrophosphate, 1 mM ATP, 0.1 mM benzamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 30 mM imidazole pH 7.0 and 5 mM dithiothreitol (DTT)] by disruption of cells with glass beads. After a low speed spin to remove cell debris and glass beads, the lysate was loaded on to a 5–20% glycerol gradient prepared in pyrophosphate buffer. The gradients were spun for 19 h in a SW50.1 rotor at 40 000 r.p.m. and fractions collected. A parallel gradient was loaded with molecular size standards.

Poly-L-proline affinity chromatography

PLP affinity chromatography was performed as described in Haarer *et al.* (1990), using buffer conditions devised in Machesky *et al.* (1994). Briefly, 200 mg of PLP were dissolved in water by gentle rocking at 4°C and coupled to CNBr-activated Sepharose beads as described by the manufacturer. Cell lysates were prepared from ~60 g of *S.pombe* cells by glass bead disruption and spun at 100 000 g for 1 h. This high speed supernatant was dialyzed into Tris–pyrophosphate buffer (10 mM Tris pH 8.0, 7.5 mM sodium pyrophosphate, 1 mM DTT and 0.6 mM PMSF) overnight and loaded on to a DEAE–cellulose matrix which

previously was equilibrated with Tris-pyrophosphate buffer. The flow-through from the ion-exchange matrix was loaded onto a PLP-Sepharose column. After extensive washes, proteins bound to the PLP-Sepharose column were eluted as described in Results and in the legend to Figure 6C.

Cell labeling and immunoprecipitation

Wild-type cells were metabolically labeled with 1 mCi of a mixture of ³⁵S-labeled methionine and cysteine (Trans label, ICN) for 4 h in minimal medium. Cells were lysed in pyrophosphate buffer and immunoprecipitated with antibodies against Sop2 or against Arp3p (McCollum *et al.*, 1996). The respective pre-immune sera were used as controls. After 1 h on ice, immune complexes (referred to as primary immune complexes) were isolated on protein A-Sepharose beads and washed extensively in pyrophosphate buffer. Immune complexes were resuspended in 100 µl of SDS lysis buffer (10 mM NaPO₄, pH 7.0, 0.5% SDS, 1 mM DTT, 1 mM EDTA, 50 mM NaF, 100 µM Na₃VO₄, 4 µg/ml leupeptin) boiled for 2 min at 95°C and diluted with 500 µl of NP-40 buffer (6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 4 µg/ml leupeptin, 100 µM Na₃VO₄, 1 mM PMSF, 2 mg/ml aprotinin, 2 mM benzamidine). Immunoprecipitations were carried out on boiled and diluted primary immune complexes using antibodies to Sop2p or Arp3p as described in the text.

Cytological methods

F-actin staining and cell wall staining were performed as described by Marks and Hyams (1985). To visualize actin cables, cells from a 2 ml culture were fixed with 3.5% formaldehyde for 1 min, washed with phosphate-buffered saline (PBS) and permeabilized with PBS containing 1% NP-40. After permeabilization, cells were washed twice with PBS and resuspended in 20–30 µl of DAPI mounting medium to which 1 µl of rhodamine-conjugated phalloidin at 0.1 mg/ml was added. Then 2–3 µl of the cells suspension were mounted for viewing. For cell stainings with antibodies, cells were fixed with a mixture of formaldehyde and glutaraldehyde or methanol, protoplasted and stained with anti-Sop2p serum or a mixture of Sop2p serum and TAT-1 antibodies as described in Moreno *et al.* (1991). We found that Sop2p immunostaining in patches and along the septa was particularly sensitive to the duration of fixation and spheroplasting. In all cases, cells were viewed in a Zeiss axioscope with the appropriate set of filters. Photographs were taken using a camera mounted on the microscope and printed on Agfa-Rapitone PI-3 paper or images captured using a Zeiss ZVS-47DEC image-capturing system.

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References

- Balasubramanian, M.K., Hirani, B.R., Burke, J.D. and Gould, K.L. (1994) The *Schizosaccharomyces pombe* *cdc3*⁺ gene encodes a profilin essential for cytokinesis. *J. Cell Biol.*, **125**, 1289–1301.
- Barbet, N., Muriel, W.J. and Carr, A.M. (1992) Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*. *Gene*, **114**, 59–66.
- Binnette, F., Bénard, M., Laroche, A., Pierron, G., Lemieux, G. and Pallotta, D. (1990) Cell-specific expression of a profilin gene family. *DNA Cell Biol.*, **9**, 323–334.
- Cooley, L., Verheyen, E. and Ayers, K. (1992) Chickadee encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. *Cell*, **69**, 173–184.
- Edamatsu, M., Hirono, M. and Watanabe, Y. (1992) *Tetrahymena* profilin is localized in the cleavage furrow. *J. Biochem.*, **112**, 637–642.
- Federov, A.A., Magnus, K.A., Graupe, H., Lattman, E.E., Pollard, T.D. and Almo, S.E. (1994) X-ray structures of isoforms of the actin binding protein profilin that differ in their affinity for phosphoinositides. *Proc. Natl Acad. Sci. USA*, **91**, 8636–8640.
- Fikes, J.D., Becker, D.M., Winston, F. and Guarente, L. (1990) Striking conservation of TFIID in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *Nature*, **346**, 291–294.
- Goldschmidt-Clermont, P.J., Kim, J.W., Machesky, L.M., Rhee, S.G. and Pollard, T.D. (1991) Regulation of phospholipase C- γ 1 by profilin and tyrosine phosphorylation. *Science*, **251**, 1231–1233.
- Goldschmidt-Clermont, P.J., Furman, M.I., Wachsstock, W., Safer, D., Nachmias, V.T. and Pollard, T.D. (1992) The control of actin nucleotide exchange by thymosin B4 and profilin. A potential regulatory mechanism for actin polymerization in cells. *Mol. Biol. Cell*, **3**, 1015–1024.
- Haarer, B.K., Lillie, S.H., Adams, A.E.M., Magdolen, V., Bandlow, W. and Brown, S.S. (1990) Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin-deficient cells. *J. Cell Biol.*, **110**, 105–114.
- Hagan, I.M. and Hyams, J.S. (1988) The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.*, **89**, 343–357.
- Haugwitz, M., Noegel, A.A., Karakesisoglou, J. and Schleicher, M. (1994) *Dictyostelium* amoebae that lack G-actin-sequestering profilins show defects in F-actin content, cytokinesis, and development. *Cell*, **79**, 303–314.
- Hoheisel, J.D., Maier, E., Mott, R., McCarthy, L., Grigoriev, A.V., Schalkwyk, L.C., Nizetic, D., Francis, F. and Lehrach, H. (1993) High-resolution cosmid and P1 maps spanning the 14Mbp genome of the fission yeast *Schizosaccharomyces pombe*. *Cell*, **73**, 109–120.
- Kelleher, J.F., Atkinson, S.J. and Pollard, T.D. (1995) Sequences, structural models, and cellular localization of the actin-related proteins Arp2 and Arp3 from *Acanthamoeba*. *J. Cell Biol.*, **131**, 385–397.
- Machesky, L.M. and Pollard, T.D. (1993) Profilin as a potential mediator of membrane-cytoskeleton communication. *Trends Cell Biol.*, **3**, 381–385.
- Machesky, L.M., Atkinson, S.J., Ampe, C., Vanderkerchove, J. and Pollard, T.D. (1994) Purification of a cortical complex containing two unconventional actins from *Acanthamoeba* by affinity chromatography on profilin-agarose. *J. Cell Biol.*, **127**, 107–115.
- Markey, F., Persson, T. and Lindberg, U. (1981) Characterization of platelet extracts before and after stimulation with respect to the possible role of profilactin as microfilament precursor. *Cell*, **23**, 145–153.
- Marks, J. and Hyams, J.S. (1985) Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *Eur. J. Cell Biol.*, **39**, 27–32.
- Maudrell, K. (1989) *nmt1* of fission yeast: a highly expressed gene completely repressed by thiamine. *J. Biol. Chem.*, **265**, 10857–10864.
- Maudrell, K. (1993) Thiamine-repressible vectors pREP and pRIP for fission yeast. *Gene*, **123**, 127–130.
- McCcollum, D., Feoktistova, A., Morphew, M., Balasubramanian, M. and Gould, K.L. (1996) The *Schizosaccharomyces pombe* actin related protein Arp3, is a component of the cortical actin cytoskeleton and interacts with profilin. *EMBO J.*, **15**, 6438–6446.
- Moreno, S., Klar, A. and Nurse, P. (1991) Molecular genetic analysis of fission yeast, *Schizosaccharomyces pombe*. *Methods Enzymol.*, **194**, 795–823.
- Neer, E.J. and Smith, T.F. (1996) G protein heterodimers: new structures propel new questions. *Cell*, **84**, 175–178.
- Neer, E.J., Schmidt, C.J., Nambudripad, R. and Smith, T.F. (1994) The ancient regulatory-protein family of WD-repeat proteins. *Nature*, **371**, 297–300.
- Nurse, P., Thuriaux, P. and Nasmyth, K. (1976) Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.*, **146**, 167–178.
- Okayama, H. and Berg, P. (1983) A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell Biol.*, **3**, 280–289.
- Olmsted, J.B. (1981) Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.*, **256**, 11955–11957.
- Pantaloni, D. and Carlier, M.-F. (1993) How profilin promotes actin filament assembly in the presence of thymosin β 4. *Cell*, **75**, 1007–1014.
- Prentice, H.L. (1991) High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. *Nucleic Acids Res.*, **20**, 621.
- Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B.M. and Walter, U. (1995) The proline-rich focal adhesion protein VASP is a ligand for profilins. *EMBO J.*, **14**, 1583–1589.
- Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene*, **67**, 31–40.

- Tanaka,M. and Shibata,H. (1985) Poly (L-proline)-binding proteins from chick embryos are a profilin and a profilactin. *Eur. J. Biochem.*, **151**, 291–297.
- Verheyen,E.M. and Cooley,L. (1994) Profilin mutations disrupt multiple actin-dependent processes during *Drosophila* development. *Development*, **120**, 717–728.
- Vinson,V.K., Archer,S.J., Lattman,E.E., Pollard,T.D. and Trochia,D.A. (1993) Three-dimensional solution structure of *Acanthamoeba* profilin I. *J. Cell Biol.*, **122**, 1277–1283.

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