Flp1, a fission yeast orthologue of the *S. cerevisiae CDC14* gene, is not required for cyclin degradation or rum1p stabilisation at the end of mitosis

Nathalie Cueille[1],*, Ekaterina Salimova[1],*, Veronica Esteban[2],*, Miguel Blanco[2],*, Sergio Moreno[2], Avelino Bueno[2] and Viesturs Simanis[1],‡

[1]Cell Cycle Control Laboratory, Swiss Institute for Experimental Cancer Research (ISREC), Chemin des Boveresses 155, 1066 Epalinges, Switzerland
[2]Instituto de Microbiologia Bioquimica, CSIC/Universidad de Salamanca, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca, Spain
*These authors contributed equally to this paper
‡Author for correspondence (e-mail: viesturs.simanis@isrec.unil.ch)

SUMMARY

In *Saccharomyces cerevisiae*, the phosphoprotein phosphatase Cdc14p plays a central role in exit from mitosis, by promoting B-type cyclin degradation and allowing accumulation of the cyclin-dependent kinase inhibitor Sic1p. Cdc14p is sequestered in the nucleolus during interphase, from where it is released at the end of mitosis, dependent upon mitotic exit network function. The *CDC14* gene is essential and loss-of-function mutants arrest at the end of mitosis. We have identified a fission yeast orthologue of *CDC14* through database searches. A *Schizosaccharomyces pombe flp1* (cdc fourteen-like-phosphatase) null mutant is viable, divides at a reduced size and shows defects in septation. flp1p is not the essential effector of the *S. pombe* septation initiation network, but may potentiate signalling of the onset of septation. In contrast to *S. cerevisiae* Cdc14p, flp1p is not required for the accumulation or destruction of the B-type cyclin cdc13p, the cyclin-dependent kinase inhibitor rum1p, or for dephosphorylation of the APC/C specificity factor ste9p in G_1. Like its budding yeast counterpart, flp1p is restricted to the nucleolus until mitosis, when it is dispersed through the nucleus. In contrast to *S. cerevisiae* Cdc14p, flp1p is also present on the mitotic spindle and contractile ring. The potential roles of flp1p in cell cycle control are discussed.

Key words: Mitosis, Cytokinesis, Phosphatase, Mitotic exit

INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae,* the phosphoprotein phosphatase encoded by the *CDC14* gene plays a central role in exit from mitosis and completion of the cell cycle (Hoyt, 2000). The *CDC14* gene is essential (Wan et al., 1992), and heat-sensitive mutants arrest with an elongated mitotic spindle, separated chromosomes and high levels of *CDC28*-CLB kinase activity (Fitzpatrick et al., 1998; Jaspersen et al., 1998). During interphase, Cdc14p is sequestered in the nucleolus, where it is bound to Net1p as part of the RENT complex (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Release of Cdc14p from the nucleolus at the end of mitosis permits increased expression and stabilisation of the *CDC28*-CLB kinase inhibitor p40^{sic1}, and the activation of APC/C^{Cdh1p}-dependent ubiquitination of B-type cyclins, thereby promoting their destruction. Together, these events reduce *CDC28*-CLB kinase activity, permitting exit from mitosis, and cytokinesis (Visintin et al., 1998).

Release of Cdc14p from the nucleolus, and exit from mitosis requires the activity of the mitotic exit network (MEN) proteins (Visintin et al., 1999). Loss-of-function MEN mutants arrest at the end of mitosis with a phenotype similar to that of *cdc14* mutants. Increased expression of *CDC14* can rescue MEN mutants, but not vice-versa, leading to the suggestion that the

essential function of the MEN is to mediate release of Cdc14p from the nucleolus (Jaspersen et al., 1998; Visintin et al., 1999). Functional homologues of Cdc14p have been identified in higher eukaryotes, although their role remains enigmatic (Li et al., 1997).

In the fission yeast *Schizosaccharomyces pombe,* the orthologues of the MEN genes control the onset of septum formation, and are collectively referred to as the septation initiation network (SIN) (Balasubramanian et al., 2000; Le Goff et al., 1999a; Sawin, 2000). Activation of the spg1p GTPase switch is central to signalling the onset of septum formation, which also involves the concerted action of four protein kinases (cdc7p, sid1p, sid2p and plo1p); cdc14p, which binds to sid1p; mob1p, which binds to sid2p; and sid4p, which acts as a scaffold for localisation of these proteins to the spindle pole body. (Note that fission yeast cdc14p bears no structural similarity to its *S. cerevisiae* namesake.) Cdc11p encodes the fission yeast orthologue of the *S. cerevisiae nud1* gene product (A. Krapp and V.S., unpublished). Mutants defective in SIN signalling make a medial F-actin ring at the onset of mitosis to mark the division site but do not make a septum at the end of mitosis, becoming elongated and multinucleated (Chang and Gould, 2000; Fankhauser and Simanis, 1993; Fankhauser and Simanis, 1994; Guertin et al., 2000; Hou et al., 2000; Nurse et al., 1976; Salimova et al., 2000; Schmidt et al., 1997; Sparks

et al., 1999). Failure to turn off SIN signalling at the end of mitosis results in multiple rounds of septum formation without cell cleavage (Cerutti and Simanis, 1999; Minet et al., 1979; Ohkura et al., 1995; Schmidt et al., 1997; Song et al., 1996). Signalling via the SIN is negatively effected by a two-component GTPase-activating protein comprised of the products of the *byr4* and *cdc16* genes (Furge et al., 1998). Both genes are essential (Fankhauser et al., 1993; Song et al., 1996). Loss of cdc16p function gives rise to two kinds of septated cell, depending upon the cell cycle stage of the inactivation: type I cells, which are binucleate, and undergo multiple rounds of septum formation after mitosis, and type II, which are mononucleate, and probably result from inactivation of cdc16p in G_1 (Minet et al., 1979). The ultimate target of the SIN remains enigmatic. However, the budding yeast paradigm suggested an attractive hypothesis, which we have tested, that it might be a phosphoprotein phosphatase analogous to Cdc14p. In this paper, we present the characterisation of *flp1*, a fission yeast orthologue of the *S. cerevisiae CDC14* gene. Our data indicate that the *flp1⁺* gene performs a different role in cell cycle progression to that of *S. cerevisiae* Cdc14p.

MATERIALS AND METHODS

Standard methods were used for manipulation of DNA (Sambrook et al., 1989). Fission yeast were grown and manipulated according to standard protocols in either yeast extract (YE) or minimal (M) medium, containing appropriate supplements (Moreno et al., 1991). The *S. pombe* strains used in this study are from the Simanis, Moreno or Bueno lab collections. The following strains were obtained from other labs: *sid4-SA1* (Kathy Gould, Nashville, TN), *sid2-250* and *sid1-239* (Dan McCollum, University of Massachusetts), *rng2-D5* (Mohan Balasubramanian, IMA, Singapore). Since deletion of *ste9⁺* or *rum1⁺* causes sterility, all the crosses involving a deletion of these genes were done by transforming with pREP3X-*ste9⁺*, pREP3X-*rum1⁺*, and the double mutants were checked subsequently to ensure that the plasmid had been lost. Yeast transformation was carried out using the lithium acetate transformation protocol (Norbury and Moreno, 1997). Induction synchrony by *cdc25-22* arrest-release was performed as described (Moreno et al., 1989). For induction of the *nmt1* promoter, a culture growing exponentially in medium containing thiamine was washed twice and resuspended in medium without thiamine. The episomal REP vectors, pDW232 and the integrating *nmt1* expression vector pINT5 have been described previously (Basi et al., 1993; Fankhauser and Simanis, 1994; Weilguny et al., 1991). Gene deletion and epitope tagging were carried out as described (Bahler et al., 1998b).

Oligonucleotides used for cloning of the *flp1⁺* gene and construction of flp1-containing plasmids

The full ORF was amplified by PCR from a genomic DNA library in pUR19 (Barbet et al., 1992), digested with *Sma*I and cloned into the *Sma*I sites of pDW232 and pREP vectors. Primers used for amplification: forward, ACTGCCCCGGGTTCGCAATTACTTGTCTGATGGA; reverse, GCTCGCCCGGGAACCAGTAATTACAGGTTTATTAAG.

The *flp1* null and C-terminally tagged (GFP and 3HA) strains were constructed by direct chromosome integration of PCR fragments generated using plasmid pFA6a-kanMX6 as a template. Forward primer for deletion: CCACCAACACCCAGGTACACAATTTAGAACTCAACCATTACGGGTTTGACGAATATAGACGAGATTCGCAATTACTTGTCTGCGGATCCCCGGGTTAATTAA.   Forward primer for tagging: GTGTTAGCATGTCATCACTTAACAATACTTCTAATGGCCGTGTTGCTAAACCTAAGCCTTCTAAAAGCCG-

GCTAATTTCTCGGATCCCCGGGTTAATTAA. Reverse primer for tagging and deletion GGTGCGCTAAATCAGGGAATATTTGTAAAGTTAATTAATGAAAAATTATGCAGGGTTGACACAGTATAATTCAAAGTTAGTGAATTCGAGCTCGTTTAAAC.

PCR fragments were gel-purified and introduced into a *leu1-32 h⁻* strain following the protocol described previously (Bähler et al., 1998). Transformants were selected on YE G418 plates (100 mg/l). Correct integration was verified by PCR and Southern hybridization for the deletion and by PCR and western blotting for tagged strains.

For creation of flp1p (C286S) the following pair of oligonucleotides were used for PCR amplification of the gene: ATTGCTGTTCATTCTAAAGCAGGGCTC, GAGCCCTGCTTTAGAATGAACAGCAAT. The presence of the desired mutation was verified by sequencing.

Antisera and tagged strains

Antisera and tagged strains permitting detection of *cdc15, cdc7, spg1, cdc14, sid1, sid2, sid4, mob1* and *ste9* have been described previously (Blanco et al., 2000; Chang and Gould, 2000; Fankhauser et al., 1995; Fankhauser and Simanis, 1994; Guertin et al., 2000; Hou et al., 2000; Moreno et al., 1990; Salimova et al., 2000; Sohrmann et al., 1996; Sohrmann et al., 1998; Sparks et al., 1999). Rabbit antiserum against *S. cerevisiae* nop1p (fibrillarin) was a gift from Susan Gasser (University of Geneva).

Microscopy, flow cytometry and determination of cell number, septation and mitotic indices

Approximately 10^7 cells were collected by centrifugation, washed once with water, fixed in 70% ethanol and processed for flow cytometry or DAPI staining, as described previously (Moreno et al., 1991). A Becton-Dickinson FACScan was used for flow cytometry. To estimate the proportion of G_1 cells we determined the percentage of cells with a DNA content less than a value midway between 1C and 2C. The mitotic index was determined by counting the percentage of anaphase cells (cells with two nuclei and without a septum) after DAPI staining. The septation index was determined by counting the percentage of cells with septum after calcofluor staining. Cell number was determined using a Casy® cell number counter. DAPI-Calcofluor staining, and staining for F-actin and tubulin staining were done as described previously (Balasubramanian et al., 1997; Hagan and Hyams, 1988; Marks et al., 1986; Moreno et al., 1991). For examination of GFP-tagged proteins in living cells, TILLvisION software (v3.3; TILL Photonics GmBH) was used to analyse data captured with an IMAGO CCD camera mounted on an Olympus IX70 microscope. Deconvolution was performed with BitPlane software. Images were assembled in Adobe PhotoShop 5.5 and PowerPoint 97. For immunofluorescence of flp1p-GFP, cells were fixed and processed as previously described (Salimova et al., 2000).

Immunoprecipitations and western blot analysis

Protein extracts were prepared from $3\text{-}5\times10^8$ cells in exponential phase, that had been collected by centrifugation and frozen on dry ice. All subsequent manipulations were done on ice or in the cold room (4°C). For immunoprecipitation, soluble protein extracts were prepared by vortexing with glass beads in HEN buffer (50 mM Hepes pH 8.0; 150 mM NaCl; 5 mM EDTA; 1 mM EGTA; 50 mM β-glycerophosphate with inhibitors: 0.1 mM sodium orthovanadate, 50 µg/ml leupeptin, 1% aprotinin; 1 mM DTT; 1 mM PMSF). Beads were washed by brief vortexing in the same buffer containing 1% NP-40. Cell extracts were clarified by two successive centrifugations. Protein concentration was measured using Bradford assay (BioRad). For each immunoprecipitation, 2-3 mg of soluble protein was incubated overnight with 10 µl of either 9E10 or 12CA5 monoclonal antibodies covalently coupled to the sepharose-Protein G beads (Sigma; P3296) (~2 µg of Ab). Beads were washed three times with 1 ml of HEN-NP-40 buffer (by pelleting in a microfuge for 5 seconds), resuspended in FRB loading buffer.
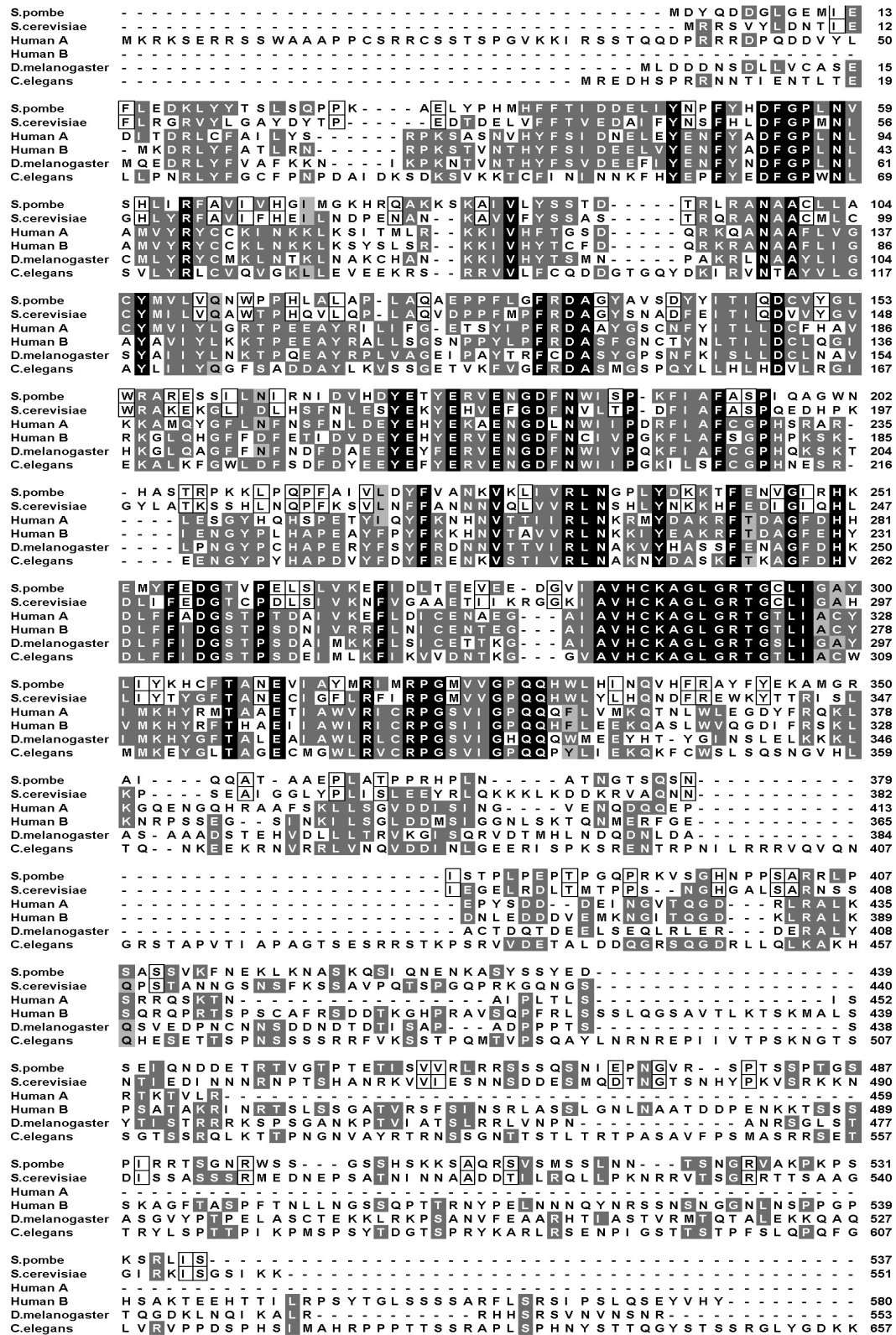
Fig. 1. Alignment of *S. cerevisiae CDC14*-related proteins. Black boxes indicate identity in all proteins, grey boxes indicate a related amino acid in three or more proteins. The following amino acids were considered interchangeable for the purposes of this alignment: K/R, S/T, I/L/V, N/Q, D/E, F/Y. When two different amino acids were present three times each, all are on a grey background, with three in white and three in black. A box indicates that the two yeast proteins have a common conserved amino acid that is not present in the other proteins. The C-terminal extension of the *C. elegans* protein is not shown.

A

B

C

D

Fig. 2. Characterisation of *flp1::kanMX6* cells. (A) The *flp1::kanMX6* allele structure. The location of the fragment used to probe the Southern blot shown in B is indicated, as are the positions of the *Eco*RI sites that give rise to the fragments observed in the autoradiograph. Correct replacement of the *flp1⁺* gene gives rise to a 2.4 kb fragment. (B) Southern blot of DNA prepared from wild-type and *flp1::kanMX6* haploid cells. Note the absence of the 3.3 kb band in the *flp1::kanMX6* cells. (C,D) *flp1::kanMX6* cells were grown in YE at 25°C to mid-exponential phase (C) and shifted to 36°C for 5 hours (D). (C) Cell 1 is a post-mitotic, binucleate cell. (D) Cell 1 is multicompartmented; cell 2 has septated once, and then undergone at least one more mitosis without septating; cell 3 has septated centrally, but both nuclei are located on the same side of the septum; and cell 4 shows one of its nuclei bisected by the developing division septum.
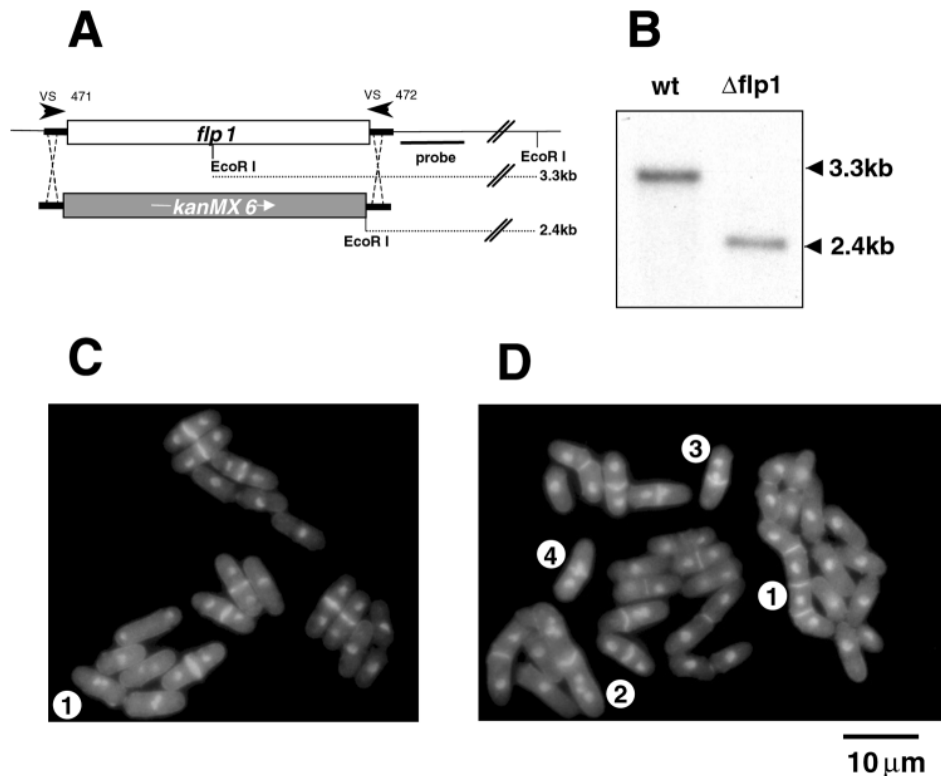
Total protein extracts were prepared from 3×10^8 cells collected by centrifugation, washed in Stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN3 pH 8.0) and resuspended in 25 μl of RIPA buffer (10 mM sodium phosphate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 150 mM NaCl, pH 7.0) containing the following protease inhibitors: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor, 100 μM 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), 100 μM *N*-tosyl-L-phenyalanine chloromethyl ketone (TPCK), 100 μM PMSF (phenylmethylsulfonyl fluoride), 1 mM phenanthroline and 100 μM *N*-acetyl-leu-leu-norleucinal. Cells were boiled for 5 minutes, broken using 750 mg of glass beads (0.4 mm Sigma) for 15 seconds in a Fast-Prep machine (Bio101 Inc.) and the crude extract was recovered by washing with 0.5 ml of RIPA. Protein concentration was determined by BCA protein assay kit (Pierce).

For western blots, 75 μg of total protein extract was run on a 12% SDS-PAGE gel, transferred to nitrocellulose and probed with rabbit affinity purified anti-ste9-C-ter (1:200), SP4 anti-cdc13 (1:250) and anti-rum1 (1:250) polyclonal antibodies. Goat anti-rabbit or goat anti-mouse conjugated to horseradish peroxidase (Amersham) (1:3,500) was used as secondary antibody. Mouse TAT1 anti-tubulin monoclonal antibodies (1:500) and goat anti-mouse conjugated to horseradish peroxidase (1:2,000) as secondary antibody was used to detect tubulin as loading control. Immunoblots were developed using the ECL kit (Amersham) or Super Signal (Pierce). CIP treatment of immunoprecipitates was performed as previously described (Fankhauser et al., 1995).

RESULTS

flp1, the fission yeast orthologue of the *S. cerevisiae* CDC14 gene is not essential for cell proliferation

A BLAST search of the fission yeast database with the S. cerevisiae *CDC14* gene open reading frame (ORF) revealed the presence of a single ORF, SPAC1782.09c, that had significant homology to Cdc14p outside the phosphoprotein phosphatase catalytic domain. A CLUSTAL-W alignment of the predicted proteins, together with putative orthologues from human, *Drosophila* and *C. elegans* is shown in Fig. 1. We have named the gene *flp1* (cdc <u>f</u>ourteen <u>l</u>ike <u>p</u>hosphatase).

To determine whether the *flp1* gene is essential for cell viability and proliferation, one copy of the gene was replaced in a diploid by the kanMX6 cassette (Fig. 2A), which was generated by PCR amplification using *flp1*-specific oligonucleotides (see Materials and Methods). Dissection of tetrads indicated that all gave rise to four colonies, of which two were resistant to Geneticin, and two were not. To confirm correct deletion of the *flp1* gene, the haploid *flp1::kanMX6* cells were checked for the absence of the *flp1⁺* gene by Southern blotting (Fig. 2B). We conclude that the *S. pombe flp1* gene is not essential for cell viability or proliferation.

Although viable, and capable of colony formation at all temperatures, *flp1::kanMX6* cells are not phenotypically identical to wild-type (Fig. 2C,D). Measurement of the length of septated cells at 25°C indicated that *flp1::kanMX6* cells divide at an average size of 11.5 μm, compared with 14 μm for wild-type. The cells are thus advanced into mitosis and may be considered 'semi-wee'. At 36°C, multi-compartmented cells (Fig. 2D, cell 1), multinucleated postmitotic cells (Fig. 2D, cell 2, C, cell 1), anucleate compartments (Fig. 2D, cell 3), and 'cut' nuclei (Fig. 2D, cell 4) were all observed. Staining with Rhodamine-conjugated Phalloidin indicated that no medial ring was present in the post-mitotic, multinucleated cells, indicating that this phenotype does not result from activation of the *S. pombe* morphology checkpoint (Liu et al., 2000). Together, these classes of aberrant cells represented approximately 8.5% of the population. Similar defects were
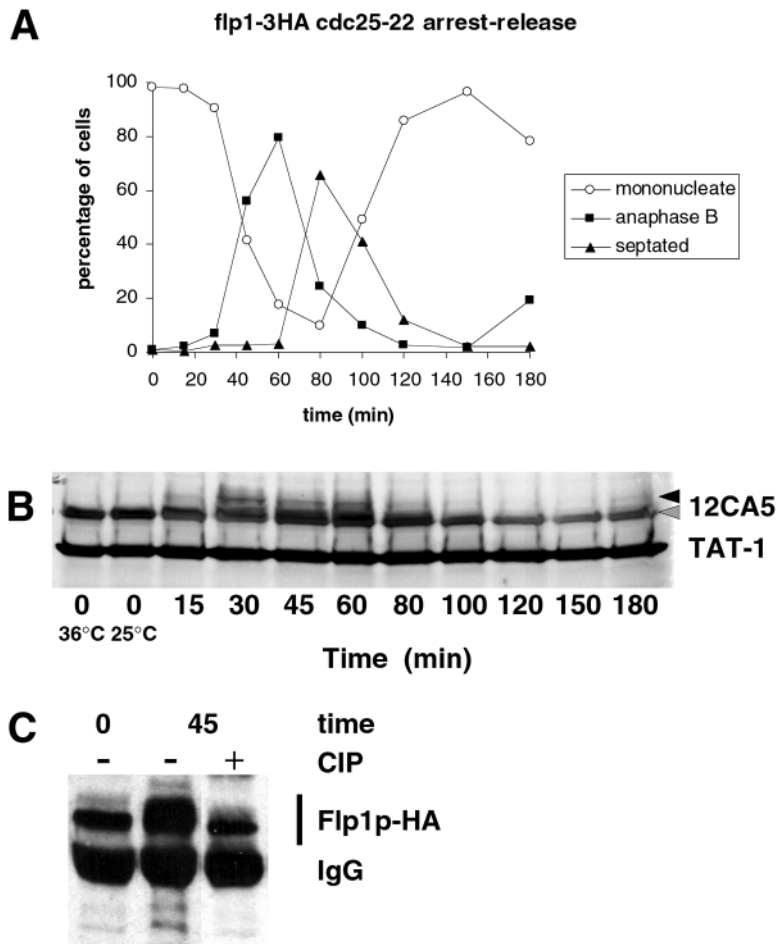
A   flp1-3HA cdc25-22 arrest-release

Fig. 3. flp1p is phosphorylated during mitosis. The strain *cdc25-22 flp1-HAc* was synchronised by arrest-release and protein samples were prepared from cells at the indicated times. Note that a slower-migrating form of flp1p-HA appears during mitosis. The black arrowhead (B) indicates the rapidly migrating, interphase form of the protein, the grey arrowhead indicates the form present in G_2 cells. (A) The progression through mitosis after release from the *cdc25-22* block, as determined by fixing cells at each time point, and staining with DAPI and Calcofluor. (B) Western blot of protein samples prepared at the indicated times, probed with 12CA5 and TAT-1, to reveal flp1p-HA and α-tubulin, respectively. (C) flp1p-HA was immunoprecipitated from protein samples prepared at the indicated times. After washing, the 45 minute immunoprecipitate was divided in half and treated with alkaline phosphatase (+), or not (−). A western blot of these protein samples was probed with 12CA5 to reveal the position of flp1p-HA.

also observed at 25°C, but these cells represented only 2% of the population. Thus, although they are viable, *flp1::kanMX6* cells display a defect in septum formation and cleavage, and are slightly advanced into mitosis.

Staining of cells with TAT-1 revealed a normal pattern of interphase microtubules and mitotic spindles (data not shown). Staining with Rhodamine-conjugated Phalloidin revealed patterns of F-actin staining similar to wild-type cells (data not shown). Examination of the SIN components cdc7p, mob1p, plo1p, spg1p and sid2p showed that their localisation was normal in *flp1::kanMX6* cells (not shown). The localisation of the medial ring components cdc15p and mid1p was also normal. Both cdc15p and mid1p undergo changes in phosphorylation during mitosis (Fankhauser et al., 1995; Sohrmann et al., 1996); these changes occurred normally in *flp1::kanMX6* cells, indicating that they are not substrates of flp1p, or that another phosphatase can substitute for flp1p in these cells (not shown).

Flp1p is phosphorylated during mitosis

Northern blotting of total RNA extracted from synchronised cells indicated that the steady state level of *flp1* mRNA does not vary significantly throughout the cell cycle (data not shown). To examine whether flp1p varies in level during the cell cycle, cells carrying the *flp1-HA* allele were synchronised by arrest release of *cdc25-22* and protein samples were prepared as cells passed through mitosis and cytokinesis

(Fig. 3A). Western blotting showed that, although the steady state level of flp1p-HA did not change significantly, there was a marked alteration in the apparent molecular weight of the protein at the time corresponding to anaphase, medial ring formation and septation (Fig. 3B, 30-80 minutes). As most cells completed cleavage and entered the next cell cycle, flp1p-HA returned to the faster-migrating form seen in G_2 arrested cells (Fig. 3B, 100-160 minutes). Flp1p-HA was immunoprecipitated from cell extracts prepared 45 minutes after release from the G_2 arrest, and the immunoprecipitate treated with alkaline phosphatase. This treatment shifted most of the protein back to the faster-migrating form (Fig. 3C), confirming that the slower-migrating forms are the result of phosphorylation.

Localisation of flp1p

To study the in vivo localisation of flp1p, the chromosomal copy of the gene was modified to add either Green Fluorescent protein (GFP) or the 12CA5 influenza virus hemagglutinin epitope tag (HA) to the C-terminus of flp1p. Both tagged proteins were considered to be functional since the tagged *flp1* allele did not show any of the strong genetic interactions shown by the *flp1::kanMX6*. Observation of both living and fixed cells demonstrated that in interphase cells the flp1p-GFP was localised in the nucleolus and on the spindle pole body (Fig. 4Aa,B,C,D). The nucleolar staining was not uniform: dots and more intensely staining regions were observed (Fig. 4Aa). The
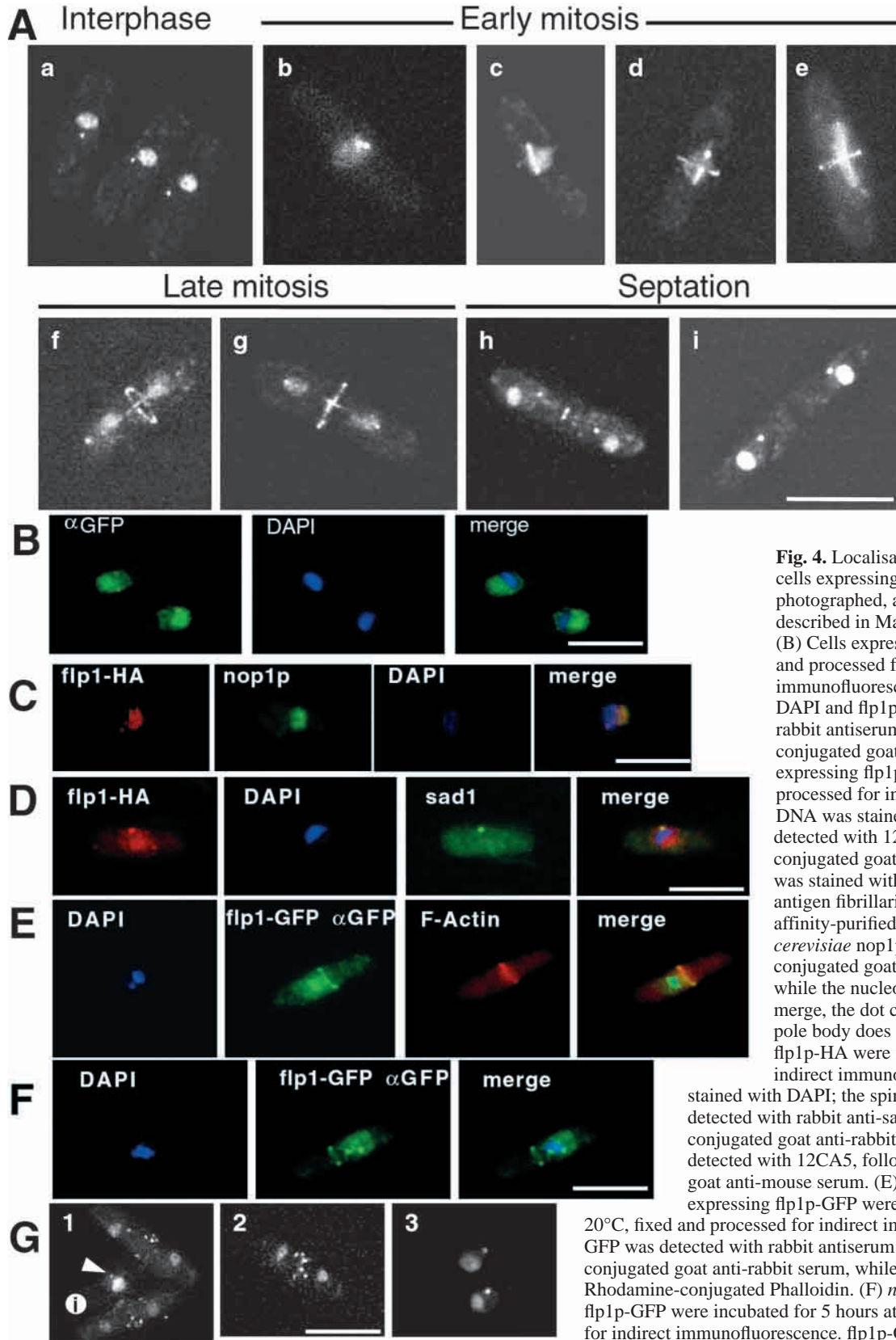
Fig. 4. Localisation of flp1p. (A) Living cells expressing flp1p-GFP were photographed, and images were processed as described in Materials and Methods. (B) Cells expressing flp1p-GFP were fixed and processed for indirect immunofluorescence. DNA was stained with DAPI and flp1p-GFP was detected with rabbit antiserum to GFP, followed by FITC-conjugated goat-anti rabbit serum. (C) Cells expressing flp1p-HA were fixed and processed for indirect immunofluorescence. DNA was stained with DAPI; flp1p-HA was detected with 12CA5 followed by CY3-conjugated goat anti-mouse serum; DNA was stained with DAPI; and the nucleolar antigen fibrillarin was detected using affinity-purified rabbit antibodies against *S. cerevisiae* nop1p followed by FITC-conjugated goat-anti-rabbit serum. Note that while the nucleolar staining overlaps in the merge, the dot corresponding to the spindle pole body does not. (D) Cells expressing flp1p-HA were fixed and processed for indirect immunofluorescence. DNA was stained with DAPI; the spindle pole antigen sad1p was detected with rabbit anti-sad1p, followed by FITC-conjugated goat anti-rabbit serum; and flp1p-HA was detected with 12CA5, followed by CY3-conjugated goat anti-mouse serum. (E) *nda3-KM311* cells expressing flp1p-GFP were incubated for 5 hours at 20°C, fixed and processed for indirect immunofluorescence. flp1p-GFP was detected with rabbit antiserum to GFP followed by FITC-conjugated goat-anti-rabbit serum, while F-actin was detected using Rhodamine-conjugated Phalloidin. (F) *nda3-KM311* cells expressing flp1p-GFP were incubated for 5 hours at 20°C, fixed and processed for indirect immunofluorescence. flp1p-GFP was detected with rabbit antiserum to GFP followed by FITC-conjugated goat anti-rabbit serum, while DNA was detected using DAPI. (G) Living cells expressing flp1p-GFP were treated with Latrunculin A for 10 minutes, then photographed. Images were processed as described in Materials and Methods. i indicates an interphase cell in image 1; the arrow indicates the location of the spindle pole body in the interphase cell. Image 2 shows the disorganised dot-like staining observed in mitotic cells, and image 3 shows a pair of interphase cells that have been treated with LatA. Note that the nucleolar and spindle pole body staining are both still present.
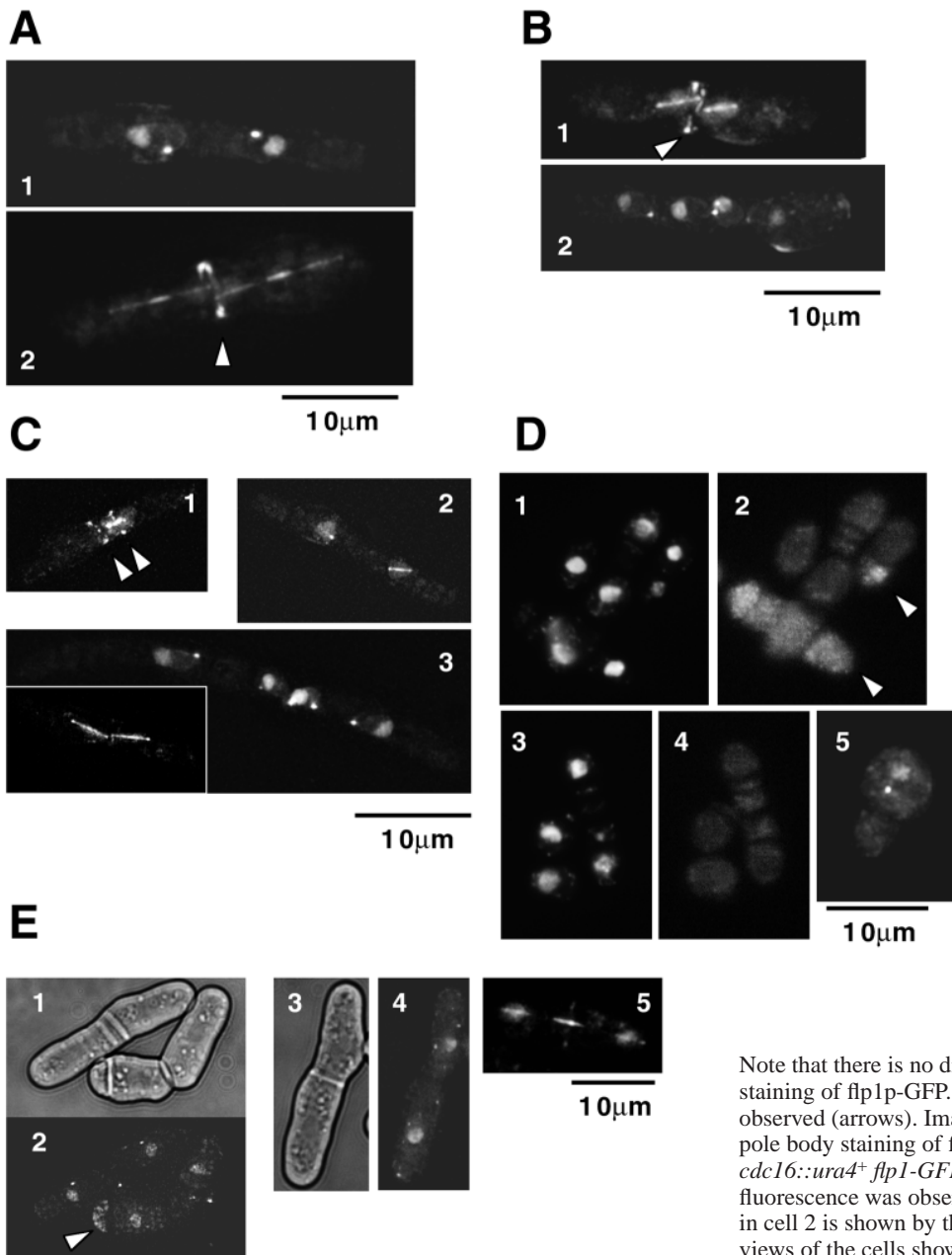
A

B

C

D

E

Fig. 5. Localisation of flp1p-GFP in SIN mutants. Spores were inoculated into minimal medium containing adenine and leucine, and allowed to germinate (except D1-4). The localisation of flp1p-GFP was examined in multinucleate cells. (A) *cdc7* null allele. (1) Interphase, binucleate cell: note that flp1p-GFP is present in the nucleolus and on the spindle pole body. (2) Cell undergoing a second mitosis: note the presence of a single medial ring and staining along the spindle. (B) *cdc14* null allele. (1) Binucleate cell, early in second mitosis: note the presence of the medial ring, and the spindle staining. (2) Multinucleate interphase cell: note that flp1p-GFP is present in the nucleolus and on the spindle pole body. (C) *spg1* null allele. (1) Mitotic cell: note the presence of two medial rings. (2) Mitotic cell: note the presence of staining along the short spindle and on the spindle pole bodies. (3) Multinucleate interphase cell: note that flp1p-GFP is present in the nucleolus and on the spindle pole body. (Inset) Binucleate cell early in the second mitosis: note the spindle staining. (D) *cdc16* null allele, germinating cells were fixed and processed for indirect immunofluorescence. flp1p-GFP was detected with rabbit antiserum against GFP (2,4); DNA was detected with DAPI (1,3). Images 1-4 are type I cells. Note that there is no discrete nucleolar or spindle pole body staining of flp1p-GFP. Spotty aggregates were sometimes observed (arrows). Image 5 is a type II cell, nucleolar and spindle pole body staining of flp1p-GFP is observed. (E) *sid2-1 cdc16::ura4+ flp1-GFPc* cells were grown at 25°C, and the GFP fluorescence was observed in living cells. The aggregate observed in cell 2 is shown by the arrow. Images 1 and 3 are phase-contrast views of the cells shown in images 2 and 4, respectively.

nature of these structures is unknown. Although the nuclear signal of flp1p-GFP was predominantly located in the non-DAPI staining (nucleolar) region of the nucleus, a weaker signal was also observed in the DAPI-staining region of the nucleus in fixed cells (Fig. 4B). The localisation to the nucleolus was confirmed by co-localisation with the nucleolar marker fibrillarin (nop1; Aris and Blobel, 1991; Henriquez et al., 1990; Potashkin et al., 1990) (Fig. 4C), while localisation to the SPB was confirmed by staining with antibodies against sad1p (Fig. 4D) (Hagan and Yanagida, 1995).

In early mitotic cells, flp1p-GFP was observed on both spindle pole bodies (Fig. 4Ab), along the mitotic spindle (Fig. 4Ac,d,e), and in the medial ring (Fig. 4Ad,e). In some cells, the staining was continuous along the short spindle (Fig. 4Ad), while in others it was discontinuous (Fig. 4Ae). The reason for this difference is presently unclear. In early mitotic cells, the

flp1p-GFP signal was present throughout the nucleus. Previous studies (Hirano et al., 1989) of the localisation of nuc1p (the large subunit of RNA polymerase I) have shown that in mitotic cells the nucleolar domain can be clearly distinguished from the DAPI-staining, chromatin region. We therefore conclude that flp1p-GFP leaves the nucleolar region early in mitosis. This is in contrast to the result observed with *S. cerevisiae* Cdc14p, which appears to leave the nucleolus only at the end of mitosis (Shou et al., 1999).

Treatment of exponentially growing *flp1-GFP* cells with Latrunculin A resulted in the appearance of centrally located flp1p-GFP dots in mitotic cells (Fig. 4G1,2). Interphase localisation to the spindle pole body and nucleolus was unaffected (Fig. 4G1i, 3). Staining of fixed *flp1::kanMX6 nda3-KM311* cells that had been arrested by incubation at 19°C, indicated that, in early mitosis, flp1p-GFP co-localised
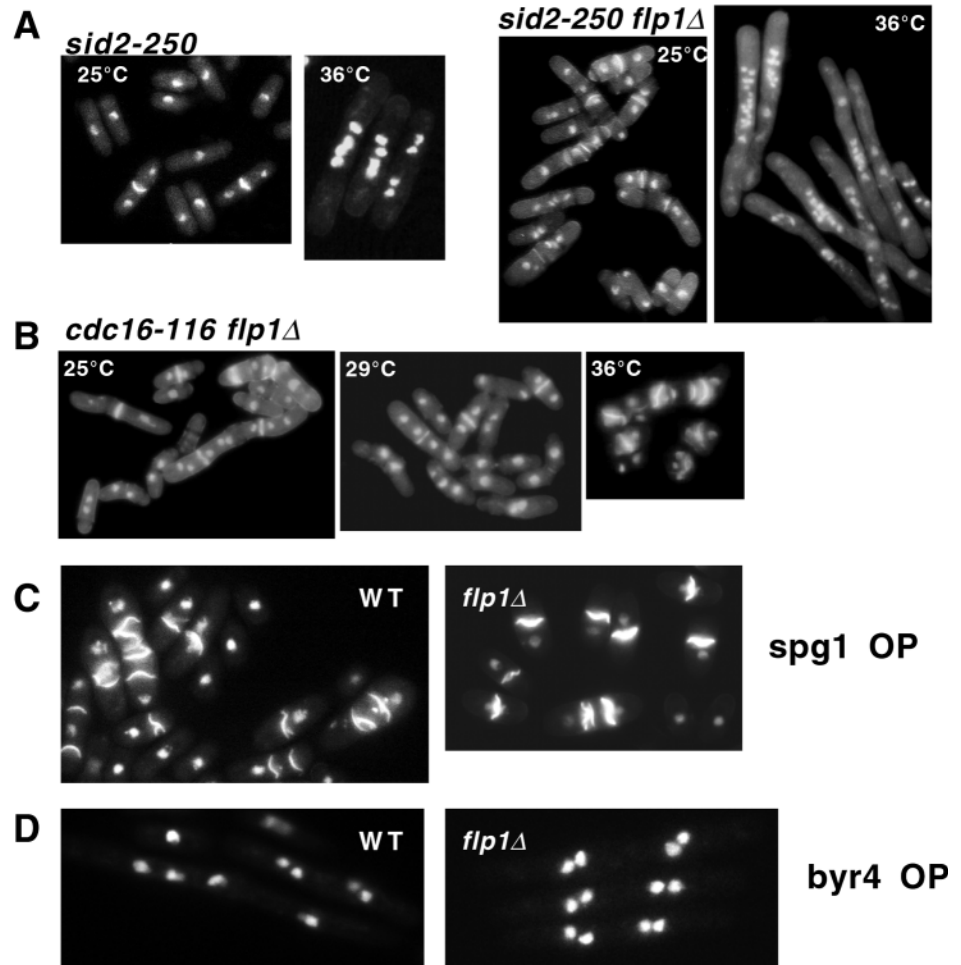
Fig. 6. Genetic interactions between the SIN and *flp1*. (A) Cells were grown in YE medium at 25°C and shifted to 36°C for 5 hours, fixed and stained with DAPI and Calcofluor. (B) Cells were grown at 29°C, then shifted for 8 hours to 25°C or for 5 hours to 36°C, before fixation and staining with DAPI and Calcofluor. (C,D) *flp1::kanMX6 leu1-32* and *leu1-32* (WT) strains were transformed to leucine prototrophy with pREP3-*spg1* (C) or pREP41-*byr4* (D). Expression of the genes was induced for 18 hours at 25°C, then cells were fixed and stained with DAPI and Calcofluor.

with the F-actin ring (Fig. 4E), and was dispersed throughout the nucleus (Fig. 4F).

In late anaphase cells, flp1p-GFP remained associated with both poles of the mitotic spindle, although the signals were weaker than in early mitotic cells (Fig. 4Af, and the medial ring (Fig. 4Af, 4Ag). The spindle staining became concentrated in the spindle mid-zone (Fig. 4Ag). During septum synthesis, flp1p-GFP was seen in the contractile ring at the leading edge of the division septum, the spindle pole bodies, and the nucleolus (Fig. 4Ah). Upon completion of the division septum (which is coincident with late G_1/S-phase in terms of the nuclear cycle), flp1p-GFP was observed uniquely on the spindle pole body and in the nucleolus, as was the case in late interphase cells (Fig. 4Ai).

Activation of the SIN is not required for flp1p-GFP relocalisation during mitosis

Previous studies have demonstrated that, in *S. cerevisiae*, MEN function is required for Cdc14p to leave the nucleolus at the end of mitosis (Shirayama et al., 1999; Shou et al., 1999; Visintin et al., 1999). To test whether SIN function is required for the cell cycle relocalisations described above, the *flp1-GFP* allele was introduced into SIN null allele backgrounds. The use of heat-sensitive SIN mutants was precluded because the spindle and medial ring staining of flp1p was found to be labile in cells fixed at high temperatures (>32°C), as has been described previously for some other SIN proteins (Salimova et

al., 2000; Sparks et al., 1999). Nucleolar and spindle pole body localisation were unaffected. The *flp1-GFP* allele was crossed to haploid SIN null strains in which the deletion is rescued by the presence of an episomal plasmid. Spores were germinated at 25°C selecting for the null allele and the *flp1-GFP* allele (see Materials and Methods). The flp1p-GFP structures present in multinucleated cells were examined. Interphase nucleolar and spindle pole body staining were observed in germinating *cdc7* null (Fig. 5A1), *cdc14* null (Fig. 5B2) and *spg1* null (Fig. 5C3) spores. Likewise, all three mutants showed spindle (Fig. 5A2,B1,C1,2) and medial ring (Fig. 5A2,5B1,5C1) staining although, in the *spg1* null cells, the rings appeared less well defined that those seen in *cdc7* null cells. Note that some SIN mutants do not always form multiple medial rings in the later nuclear cycles after shift (see for example, Balasubramanian et al., 1998; Schmidt et al., 1997), explaining why in some cases only a single ring is seen. Similar results were obtained using thermosensitive alleles of *sid2* and *sid1* that die at 29°C (not shown). We therefore conclude that SIN signalling is not required for the changes in subcellular localisation of flp1p-GFP.

Inactivation of SIN signalling is required for flp1p-GFP relocalisation to the nucleolus at the end of mitosis

The effect of deregulation of SIN signalling upon flp1p-GFP localisation was also examined. Inactivation of cdc16p, which
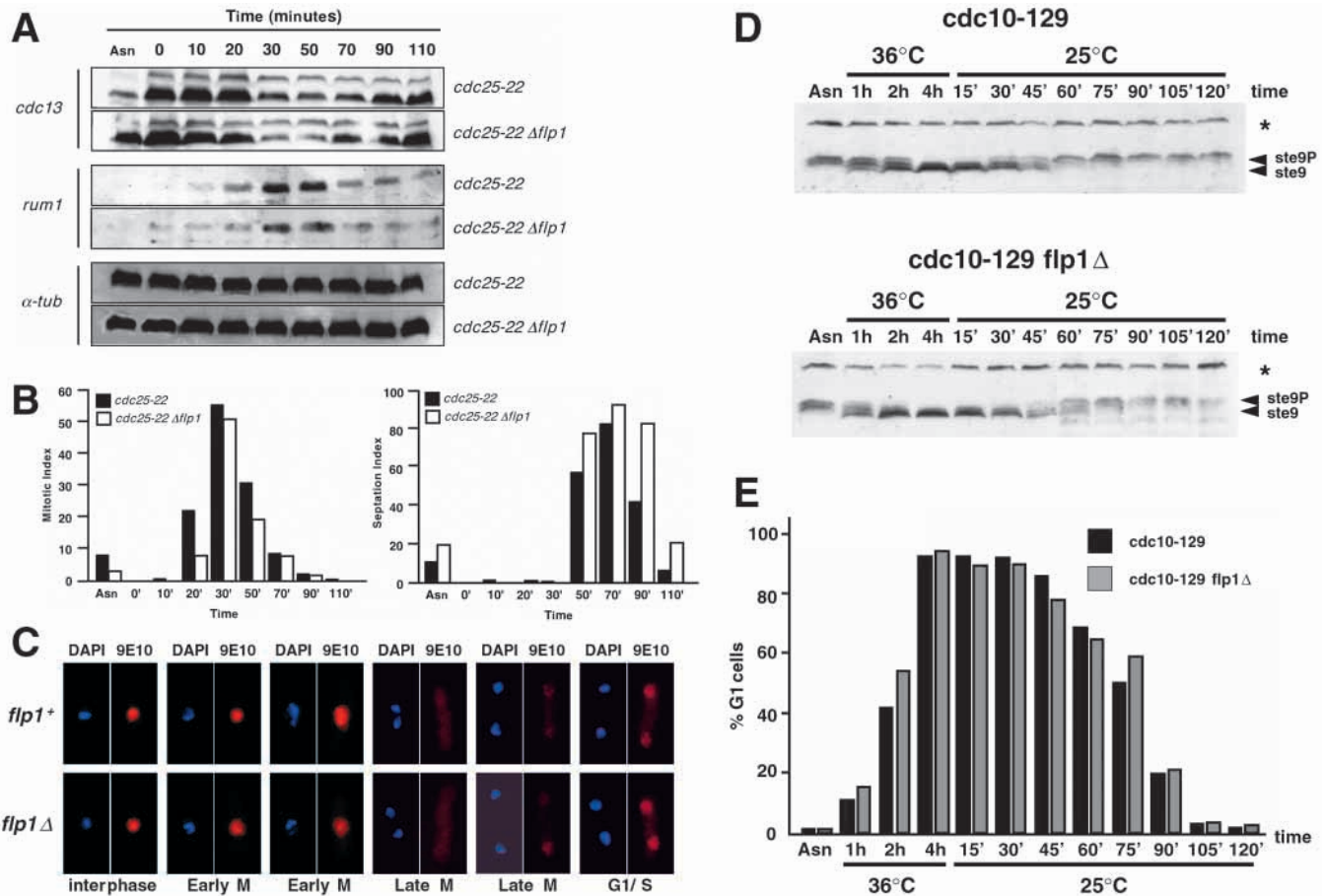
Fig. 7. flp1p function is not required for the accumulation or degradation of either rum1p pr cdc13p, or the dephosphorylation of ste9p in G₁. (A,B,C) *cdc25-22* or *cdc25-22 flp1::kanMX6* cells were synchronised by arrest-release, and protein samples were prepared at the indicated times. Western blots were probed with antiserum recognising rum1p, cdc13p and α-tubulin. (A) Western blot for rum1p, cdc13p and α-tubulin. Asn indicates asynchronous population. (B) Samples were removed at intervals, and cells were fixed, and stained with DAPI and Calcofluor. The graphs show the mitotic index and septation index at the indicated times after release from the *cdc25-22* arrest. (C) Exponentially growing *cdc13-myc13c* and *cdc13-myc13c flp1::kanMX6* cells were fixed and indirect immunofluorescence was used to detect cdc13p (mAb 9E10, followed by CY3-conjugated goat anti-mouse serum), in mitotic cells and septating cells. Note that cdc13p is destroyed and reappears with similar kinetics in the two strains. (D,E) *cdc10-129* and *cdc10-129 flp1::kanMX6* cells were grown at 25°C to mid-exponential phase in minimal medium and were shifted to 36°C for 4 hours to block the cells in G₁, and then returned to 25°C by rapid agitation in an ice-water bath. Samples were taken at the indicated times, and the ste9p phosphorylation level was examined by western blotting. (E) Samples were removed from the each culture at the indicated times, and the percentage of G₁ cells in each culture was determined by FACS analysis. The asterisk in D indicates the position of the loading control, α-tubulin, which was detected using TAT-1.

is part of the GTPase activating protein (GAP) for spg1p (Furge et al., 1998) results in multiple rounds of septum formation without cell cleavage (Minet et al., 1979). In germinating *cdc16* null spores, type II cells showed an interphase-like localisation of flp1p-GFP (Fig. 5D5). By contrast, type I cells showed no discrete localisation of flp1p-GFP (Fig. 5D1-4). Occasionally, one or more of the cell compartments contained what appeared to be aggregates of flp1p-GFP (Fig. 5D2, arrows).

Since type I cells have entered mitosis, whereas type II cells have not (see Introduction), one explanation for these data could be that once cells have entered mitosis, SIN signalling must be attenuated for flp1p-GFP to return to the interphase configuration once septation has been completed. To test this hypothesis, the localisation of flp1p-GFP was examined in cells where the lethality of a *cdc16* null allele is rescued by attenuation of the SIN (Fournier et al., 2001; Salimova et al.,

2000). The double mutant *sid2-1 cdc16::ura4⁺* is viable at 25°C, although some errors of septum formation still occur (Fournier et al., 2001). Normal interphase (Fig. 5E1-4), and mitotic (Fig. 5E5) distribution of flp1p-GFP was observed, although occasional aggregates of the protein were also seen (Fig. 5E2, arrows). A normal interphase distribution of flp1p-GFP was also seen in cells that had formed a second division septum (Fig. 5E1,2) indicating that the presence of a additional septa does not *per se* inhibit flp1p-GFP localisation in a SIN-independent manner. Together, these data are consistent with the hypothesis that SIN signalling must be inactivated or attenuated for flp1p-GFP to return to the nucleolus after septation is completed.

Genetic interactions of *flp1* with the SIN

In *S. cerevisiae*, Cdc14p is thought to be the essential target of the MEN (Jaspersen et al., 1998; Visintin et al., 1999). To

Fig. 8. Increased expression of *flp1+* arrests cells in G2. A single copy of the *flp1+* gene was integrated at the *leu1* locus of wild-type cells. Expression of *flp1+* was induced by incubation in medium without thiamine at 25°C. Samples were removed at intervals, processed for FACS analysis, and fixed and stained with Calcofluor and DAPI. (A) Uninduced control cells (top), cells 20 hours after induction (bottom). (B) FACS analysis of uninduced (top) and induced (bottom) cells. The position of the G1 peak was established by incubating cells in 12 mM hydroxyurea (HU) for 3 hours. −N are cells grown in medium lacking a nitrogen source. (C) *leu1-32* cells were transformed to leucine prototrophy with a REP3 plasmid expressing flp1p(C286S). Expression was induced for 24 hours at 25°C, cells were fixed, and stained with DAPI and Calcofluor. (D) *leu1-32* cells were transformed to leucine prototrophy with a pREP3-*flp1+*. Expression was induced (−T) and samples were removed at the indicated times thereafter. Protein extracts were prepared and western blots probed for the indicated antigens. For cdc25p, the two arrows indicate the position of the upper (hyper-phosphorylated) and lower bands. A portion of culture resuspended in medium containing thiamine served as a control. (E) *leu1-32 cdc25-HA3* was transformed with either pREP3 or pREP3-*flp1+* and expression was induced for 20 hours at 25°C. Protein samples were prepared and a western blot was probed with 12CA5. The samples were run in adjacent lanes, on the same gel.
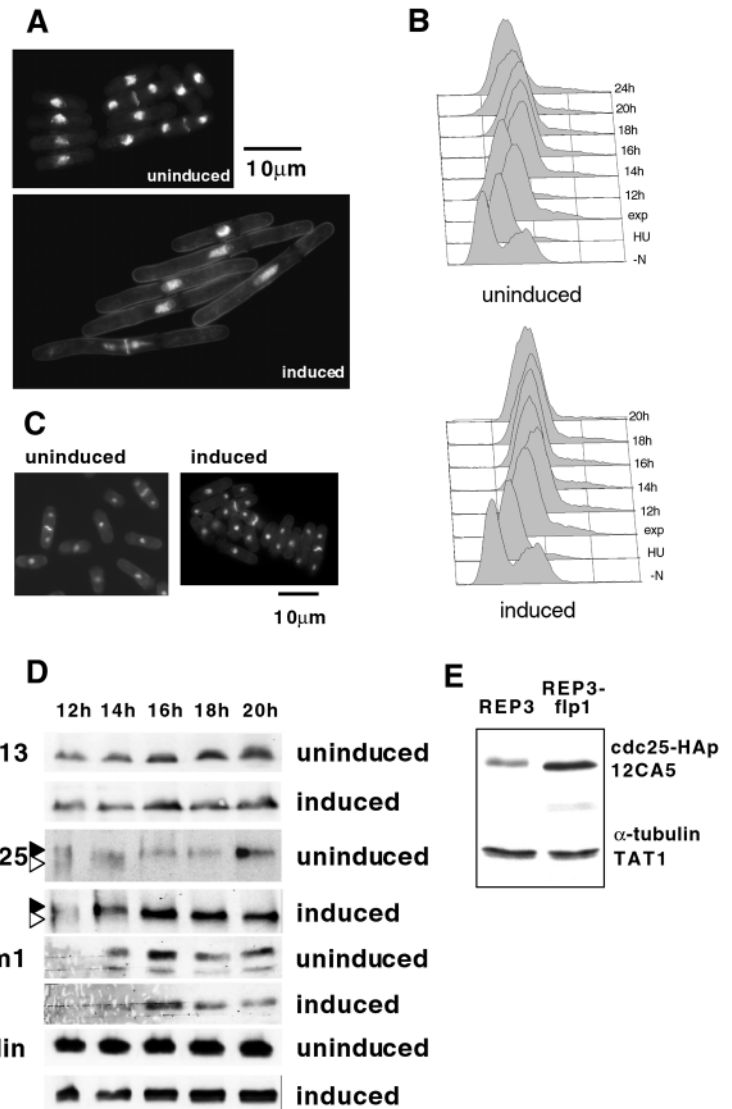
investigate whether there were any interactions between *flp1* and the SIN, double mutants of *flp1::kanMX6* with heat-sensitive SIN mutants were constructed by tetrad dissection. It was found that the double mutants *flp1::kanMX6 cdc7-24* and *flp1::kanMX6 mob1-R4* were inviable at 25°C. A strong reduction of restrictive temperature was noted for other double mutant combinations (see Table 1). In most cases, the double mutants already showed a significant septation defect at the permissive temperature: the example of the mutant *sid2-250 flp1::kanMX6* is shown in Fig. 6A. In addition, at 36°C, the double mutants accumulated more nuclei than the single SIN mutant, and the nuclear cycles were no longer synchronised (Fig. 6A). The exception to this among the SIN mutants was the double mutant *flp1::kanMX6 sid1-239*, which did not display any additive effects. Interestingly, many multinucleated, non-septated cells were observed when the double mutant *cdc16-116 flp1::kanMX6* was grown at 25°C. This defect was alleviated partially at 29°C, and at 36°C cells showed the multiseptated phenotype characteristic of cdc16-116 (Fig. 6B). No additive effects were observed when double mutants of *flp1::kanMX6* with a number of mutants defective in medial ring assembly or function were analysed (Table 1).

Increased expression of the *S. cerevisiae CDC14* can rescue some of the MEN mutants (Jaspersen et al., 1998; Visintin et al., 1999). By contrast, increased expression of the *flp1* gene from a multicopy plasmid did not rescue any of the heat-

Table 1. Effect of restrictive temperature on *S. cerevisiae* mutants

Mutant	Effect upon restrictive temperature
cdc7-24 flp1::kanMX6	Synthetically lethal at 25°C
cdc11-136 flp1::kanMX6	Reduced restrictive temperature
cdc14-118 flp1::kanMX6	Reduced restrictive temperature
spg1-B8 flp1::kanMX6	Strongly reduced restrictive temperature
sid1-239 flp1::kanMX6	No additive effect
sid2-250 flp1::kanMX6	Strongly reduced restrictive temperature
sid4-SA1 flp1::kanMX6	Reduced restrictive temperature
mob1-R4 flp1::kanMX6	Synthetically lethal at 25°C
cdc16-116 flp1::kanMX6	Elongated at 25°C, normal at 29°C, multiseptate at 36°C
ste9::ura4+ flp1::kanMX6	No additive effect
rum1::ura4+ flp1::kanMX6	No additive effect
cdc15-140 flp1::kanMX6	No additive effect
rng2-D5 flp1::kanMX6	No additive effect
cdc4-8 flp1::kanMX6	No additive effect

sensitive SIN mutants tested (*cdc7-24*, *cdc11-136*, *cdc14-118*, *spg1-B8*, *sid1-239*, *sid2-250*, *sid4-SA1*, *mob1-R4* and *cdc16-116*), whether expressed at low (from its own promoter in pDW232, or pREP3 non-induced), or high levels (pREP3, induced) (data not shown).

Increased expression of some SIN genes can induce septation independently of entry into mitosis (Ohkura et al.,

Fig. 9. Mitotic control mutants have altered sensitivity to increased expression of *flp1+*. A single, *nmt1*-promoter-controlled copy of *flp1+* was introduced into the *leu1* locus and crossed into the indicated strain backgrounds. Expression of *flp1+* was induced and cell number was determined at intervals thereafter. Cells were grown at 25°C. (A) The effect of increased *flp1+* expression in *wee1-6* and *cdc25::ura4+ wee1-50*. Induced images were taken 26 hours (approximately 6 generations) after induction. (B) Cell number increase after induction of *flp1+*. Cell numbers are expressed in arbitrary units. Cell numbers at 16 hours were approximately 10^6 ml^{-1}. (C,D) Induction of *flp1+* in dominant activated alleles of *cdc2*.

1995; Schmidt et al., 1997). Expression of either *plo1* (not shown) or *spg1* (Fig. 6C) from the nmt1 promoter induced septation in *flp1::kanMX6* cells. Likewise, increased expression of septation inhibitor *byr4* (Song et al., 1996) blocked septum formation in *flp1::kanMX6* cells, as it does in wild-type (Fig. 6D). Thus, we conclude that flp1p is not an essential effector of spg1-mediated signal transduction. However, it was noted that increased expression of *spg1* induced fewer multiseptated cells in a *flp1::kanMX6* background (Fig. 6C), suggesting that the efficiency of SIN signalling was reduced in the absence of flp1p function.

Flp1p is not necessary for dephosphorylation of ste9p in G_1, nor is it required for degradation, or accumulation of cdc13p or rum1p at the end of mitosis

S. cerevisiae Cdc14p promotes degradation of B-type cyclins at the end of mitosis, and also favours accumulation of p40^{sic1} (Visintin et al., 1998). The effects of *flp1::kanMX6* upon degradation of the B-type cyclin cdc13p and accumulation of rum1p were investigated by arrest-release of *cdc25-22* and *cdc25-22 flp1::kanMX6* strains, extraction of proteins, and western blotting. Western blotting with antisera recognising either cdc13p or rum1p indicated that both proteins accumulated and were then degraded in the *flp1::kanMX6* background with similar timing to that seen in *flp1+* cells (Fig. 7A). The kinetics of release from the *cdc25-22* block were similar in the *flp1+* and *flp1::kanMX6* backgrounds (Fig. 7B). The timing of disappearance of cdc13p-myc13 was also examined in exponentially growing *flp1+* and *flp1::kanMX6* cells. Fixed cells were stained with 9E10 and the fluorescence due to cdc13p compared in cells at similar stages in mitosis. No significant differences were noted between the *flp1+* and *flp1::kanMX6* backgrounds (Fig. 7C). We conclude that flp1p activity is not required for accumulation or degradation of either cdc13p or rum1p. Consistent with this, the *flp1::kanMX6* cells are capable of mating.

The APC/C accessory factor Cdh1p is thought to be dephosphorylated by Cdc14p at the end of mitosis, thereby allowing it to associate with the APC/C to promote B-type cyclin degradation (Jaspersen et al., 1999; Visintin et al., 1998). The *S. pombe* homologue of Cdh1p, ste9p (Kitamura et al., 1998; Yamaguchi et al., 1997), is also dephosphorylated and activated in G_1 (Blanco et al., 2000; Yamaguchi et al., 2000). Cells were synchronised by arrest-release of a *cdc10* mutant, and the phosphorylation state of ste9p was monitored by western blotting. Ste9p was dephosphorylated normally in G_1 arrested cells, and then rephosphorylated upon S-phase entry in both *flp1+* or *flp1::kanMX6* backgrounds (Fig. 7D). The kinetics of entry into S-phase following release of the *cdc10-129* block were similar in a *flp1+* and *flp1::kanMX6* background (Fig. 7E). We conclude that flp1p is not responsible for dephosphorylation of ste9p in G_1.

Increased expression of *flp1* arrests cells in G_2

Strong expression of *S. cerevisiae CDC14* promotes mitotic exit, B-type cyclin degradation, accumulation of the CKI p40^{sic1}, and G_1 arrest (Visintin et al., 1998). Increased expression of *S. pombe flp1* from the thiamine-regulated *nmt1* promoter produced elongated cells with a single nucleus (Fig. 8A). FACS analysis indicated that cells arrested
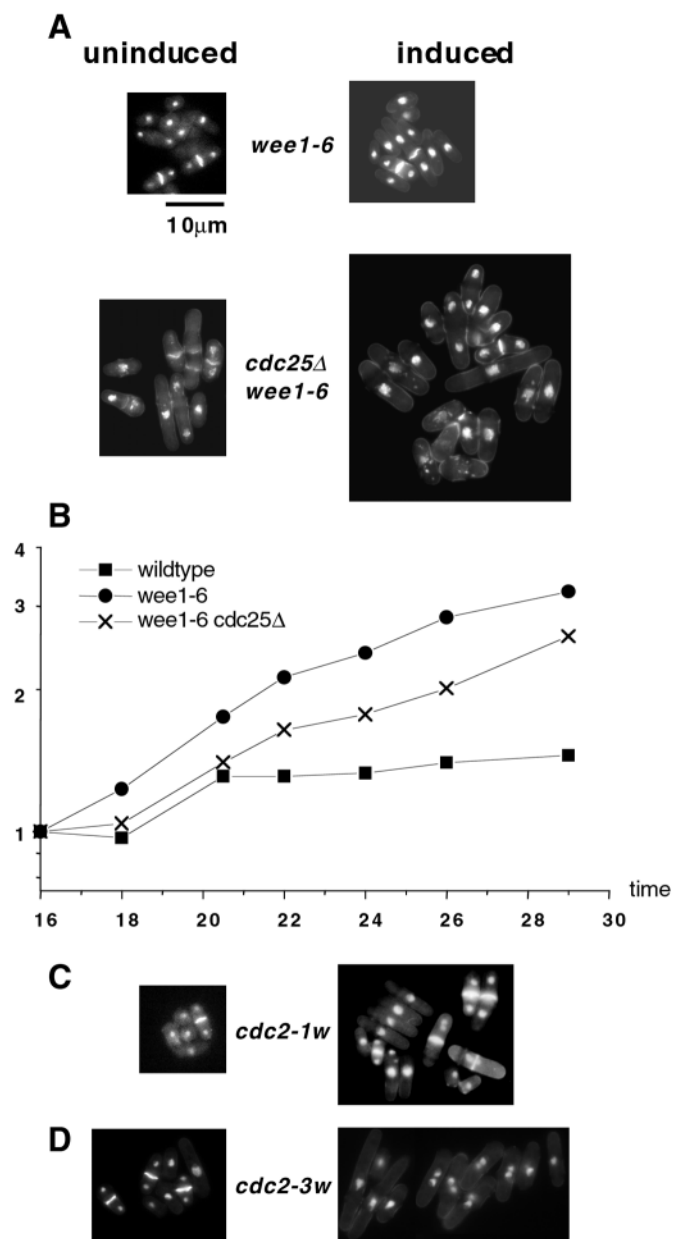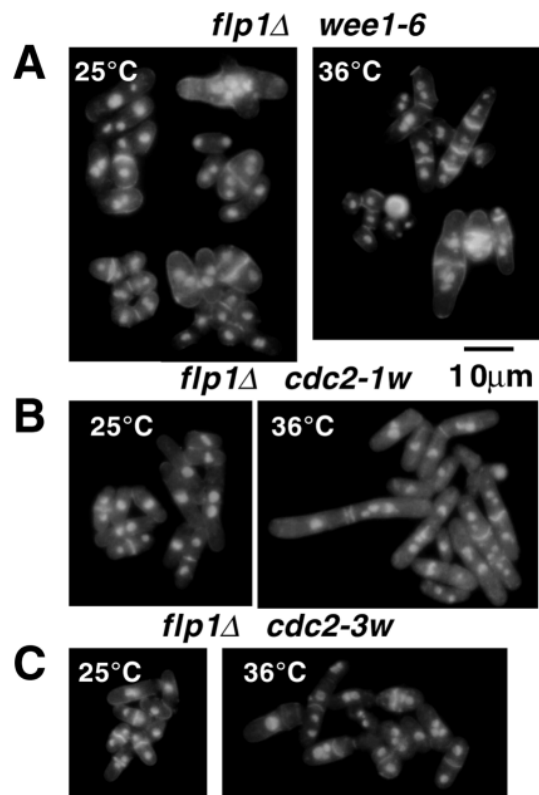
Fig. 10. Genetic interactions between *flp1::kanMX6* and mitotic control mutants. Cells of the indicated genotype were grown at 25°C in YE and a part of the culture was shifted to 36°C for 5 hours. Cells were fixed and stained with DAPI and Calcofluor.

predominantly with 2C DNA content (Fig. 8B). Staining with Rhodamine-conjugated Phalloidin indicated that F-actin patches were located at the tips of the cell consistent with a G_2 arrest (data not shown). Upon prolonged incubation, cells eventually entered mitosis and septated. Increased expression of the mutant flp1p(C286S), in which the conserved cysteine that is known to be essential for activity of *S. cerevisiae* Cdc14p (Taylor et al., 1997) is replaced by serine, did not produce a cell cycle arrest, indicating that the phosphatase activity of flp1p is essential for cell cycle arrest (Fig. 8C). Western blotting showed that the levels of the mitotic B-type cyclin cdc13p and the cdk-inhibitor rum1p did not change significantly in the arrested cells (Fig. 8D), indicating that the arrest does not result from degradation of B-type cyclins or stabilisation of rum1p. The mitotic inducer cdc25p was shifted to a faster-migrating form (Fig. 8D). This was confirmed by expressing *flp1* in a strain carrying a myc-tagged cdc25p (Fig. 8E), and suggesting that the G_2 arrest might be mediated in part through the mitotic regulatory system.

A cell cycle block was also imposed by increased expression of *flp1* in *rum1::ura4+*, *ste9::ura4+* and *rum1::ura4+ ste9::ura4+* mutants, indicating that neither ste9p nor rum1p is necessary for the cell cycle arrest induced by increased expression of flp1p. Expression of *flp1* in *rad3−*, *chk1Δ*, and *rad24Δ* backgrounds also blocked cell division, suggesting that the G_2 arrest does not result from ectopic activation of the DNA structure checkpoint (data not shown).

Mitotic control mutants prevent cell cycle arrest in response to increased expression of *flp1+*

The effects of increased *flp1* expression were studied in the mutants *wee1-6* (which divide at a reduced size at all temperatures (Fantes, 1981) and *cdc25::ura4+ wee1-6* (which lacks the normal size control over entry into mitosis (Sveiczer et al., 1999). Both strains continued dividing at times following induction of *flp1+* expression when wild-type cells had arrested (Fig. 9A,B). This demonstrates that the G_2 arrest following increased expression of flp1p requires the mitotic inhibitor wee1p.

The effect of increased *flp1+* expression in the presence of activated alleles of *cdc2* was also investigated. The mutant *cdc2-1w* is less responsive than *cdc2+* to inhibition by wee1p, but still requires cdc25p for activation (Fantes, 1983; Russell and Nurse, 1987b). Increased expression of *flp1+* in *cdc2-1w* resulted in some cell elongation, but septated, dividing cells were still present, indicating that, as in the *wee1-6* background, the cell cycle arrest was relieved. Moreover, anucleate compartments and cut nuclei were observed, indicating a loss of proper co-ordination between mitosis and septum formation (Fig. 9C). The mutant *cdc2-3w* is independent of cdc25p for activation, but still responds to inhibition by wee1p (Russell and Nurse, 1987b). In this background, increased expression of *flp1+* arrested cell division. Approximately 50% of cells had a single nucleus, while the remainder had two, whose chromatin domains faced each other, indicating that these cells had completed mitosis without septating (Fig. 9D). Together, these data suggest that the G_2 arrest due to *flp1* overexpression is imposed predominantly through the mitosis regulators cdc25p and wee1p.

Genetic interactions of *flp1::kanMX6* with mitotic control and other mutants

The genetic interactions of *flp1::kanMX6* with elements of the mitotic control system were also examined. Double mutants with *wee1-6*, *cdc2-1w*, and *cdc2-3w* were constructed. The mutants *wee1-6 flp1::kanMX6*, *cdc2-1w flp1::kanMX6* and *cdc2-3w flp1::kanMX6* showed strong additive effects. Multinucleate cells were frequently observed, indicating that cells had failed to septate. In addition, enlarged nuclei and nuclei of different sizes in the same compartment were observed, suggesting that aberrant mitoses had occurred. These phenotypes were more accentuated at 36°C than 25°C (Fig. 10A,B,C). The mutant *cdc2-3w* is defective in sensing the completion of DNA replication (Enoch and Nurse, 1990). Incubation of *cdc2-3w flp1::kanMX6* in medium containing hydroxyurea produced 'cut' phenotypes at both 25°C and 36°C, indicating that the *flp1::kanMX6* mutation does not restore the DNA replication completion checkpoint function. Consistent with this, *flp1::kanMX6* cells arrested normally in the presence of hydroxyurea, indicating that the DNA replication completion checkpoint is functional (data not shown). No additive effects were noted in the double mutants *rum1::ura4+ flp1::kanMX6* and *ste9::ura4+ flp1::kanMX6* (Table 1; data not shown).

Flp1p is required for the nuclear division delay imposed by the *S. pombe* morphology checkpoint

Defects in septum construction can trigger a checkpoint that delays the resumption of tip-growth, and disassembly of the
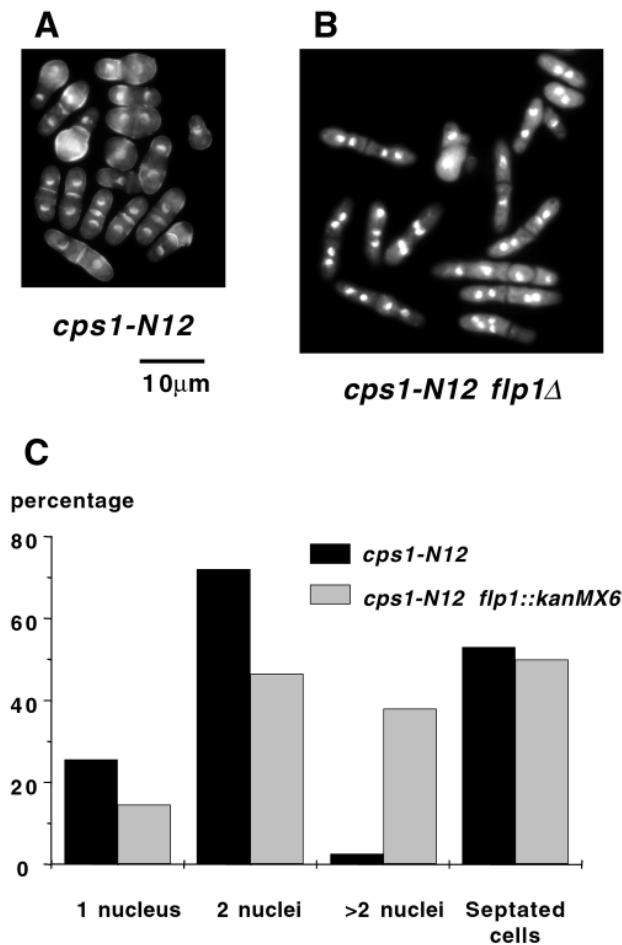
Fig. 11. *flp1⁺* functions in the *S. pombe* morphology checkpoint. Cells of the indicated genotype were grown at 25°C in YE, then shifted for 5 hours to 36°C before fixation. (A,B) DAPI and Calcofluor staining of cells 5 hours after shift to 36°C. (C) The percentage of cells with one, two or more than two nuclei. Cells were scored as 2 or >2 nuclei, whether they were septated or not, as long as there was no sign of cleavage of the septum.

medial F-actin ring. This delay requires both wee1p and SIN function (Le Goff et al., 1999b; Liu et al., 2000). Since the *flp1::kanMX6* allele showed strong genetic interactions with the SIN and mitotic control genes, the effect upon the morphology checkpoint was examined by constructing the double mutant *cps1-N12 flp1::kanMX6*. The *cps1-N12* mutant is defective in β-glucan synthase function (Le Goff et al., 1999b). After shift to 36°C, more than 70% of cells arrest binucleate, with or without a division septum, having activated the morphology checkpoint (Fig. 11A,C). By contrast, although the aberrant septa were not cleaved, the double mutant *cps1-N12 flp1::kanMX6* became elongated, and many cells underwent a second round of nuclear division during the incubation period (Fig. 11B,C). We conclude that flp1p function is required for the *S. pombe* morphology checkpoint.

DISCUSSION

In this paper we have presented the characterisation of *flp1*, the

S. pombe orthologue of the *S. cerevisiae CDC14* gene. Although *flp1* has some features in common with its *S. cerevisiae* counterpart, its role in cell cycle control seems to be significantly different.

Why is flp1p function not essential ? At the time of writing, while the sequence of the fission yeast genome remains incomplete (though only about 50 gene's-worth of DNA remains to be sequenced), it is not possible to exclude formally that there is a second flp1p-like protein in fission yeast, with which flp1p is functionally redundant. Nonetheless, database searches do not identify any other closely related protein, and Southern blotting has not identified any closely related sequences.

Both proteins are nucleolar-located in interphase, from where they are released during mitosis. However, while release of *S. cerevisiae* Cdc14p from the nucleolus requires MEN function, the data presented in this paper indicate clearly that SIN function is not required to effect flp1p release from the nucleolus in *S. pombe*. In budding yeast, Cdc14p is tethered in the nucleolus by attachment to Net1p, which is a component of the RENT complex (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Database searches have failed to identify a Net1p orthologue in *S. pombe* to date. In this context, it is noteworthy that the closest *S. pombe* homologue of Sir2p (ORF SPBC16d10.07), which is another member of the RENT complex in *S. cerevisiae*, is also located in the nucleolus (M. Cockell, V.S. and S. Gasser, unpublished).

What is the signal for release of flp1p from the nucleolus? Flp1p is phosphorylated during mitosis, at the time when it is associated with the medial ring, the mitotic spindle, and is found throughout the nucleus. It is tempting to speculate that the phosphorylation is the trigger for the relocalisation of flp1p. It is noteworthy that there are two cdc2p consensus sites (S/TPXK/R) in flp1p. However, these are not conserved in the Cdc14p orthologues from other species. The role of phosphorylation in the regulation of flp1p will be the subject of future studies.

In *S. pombe*, return of flp1p to the nucleolus at the end of mitosis requires inactivation or attenuation of the SIN. It is possible that inactivation of SIN signalling marks the successful completion of a division septum and that. while it is active, it ensures that the flp1p remains available to dephosphorylate a substrate that is important to permit septation and/or cell cleavage. It is unlikely that initiation of DNA synthesis is the trigger for nucleolar localisation of flp1p at the end of mitosis, since a *cdc16-116* mutant undergoes DNA synthesis at the non-permissive temperature (Minet et al., 1979).

Does flp1p play a role in controlling the onset of mitosis ?

Cells lacking flp1p function are advanced into mitosis, suggesting that flp1p is an inhibitor of mitosis. Consistent with this, increased expression of *flp1⁺* arrests cells in G_2, in a wee1p-dependent manner. In the arrested cells, cdc25p is in a rapidly migrating, dephosphorylated form. Since cdc25p is activated by phosphorylation during mitosis (Creanor and Mitchison, 1996; Ducommun et al., 1990; Moreno et al., 1990), it is possible that this contributes significantly to the G_2 arrest. The fact that *flp1* overexpression still marrests the cell cycle in a cdc25p-independent manner in the *cdc2-3w* mutant, suggests

that it also acts upon either wee1p, or one of its regulators, such as nim1p (Russell and Nurse, 1987a; Wu and Russell, 1993), nif1p (Wu and Russell, 1997), or cdr2p (Breeding et al., 1998; Kanoh and Russell, 1998) to delay mitotic entry. In this context, it is noteworthy that cdr2p has been implicated in regulating septum formation, although at which level is not clear (Breeding et al., 1998).

In *S. pombe*, both cdc25p and wee1p are nuclear proteins, found predominantly, although not exclusively, in the DAPI-staining region of the nucleus (Aligue et al., 1997; Wu et al., 1996; Zeng and Piwnica-Worms, 1999). It is also noteworthy that some flp1p-GFP is observed in the non-nucleolar part of the nucleus. It is therefore possible that during interphase, cdc25p and wee1p phosphorylation states are modulated by flp1p to maintain them in an inactive and active state, respectively. Whether this is direct or indirect remains to be determined. Thus, although flp1p function is not essential for entry into (or exit from) mitosis, flp1p may play a 'fine-tuning' role in regulating entry into mitosis. The fact that flp1 function is not essential either for mitotic onset or completion may be due to the presence of multiple control systems, which can compensate for each other.

How does flp1p function in controlling the onset of septation?

It has been suggested that *S. cerevisiae* Cdc14p is the essential effector of the MEN. By contrast, flp1p is not an essential effector of SIN signalling. Nonetheless, SIN mutants that appear wild-type at 25°C in a *flp1+* background show a strong septation phenotype in the absence of flp1p function. It is therefore possible that one of the roles of flp1p is to potentiate SIN signalling, for example, by activating one or more elements of the network. Two observations are consistent with this: first, *flp1::kanMX6* cells show defects in septation signalling, and second, induction of septation by *spg1* overexpression is less efficient in the *flp1::kanMX6* background, suggesting that the absence of flp1p attenuates SIN signalling. In this context, it is noteworthy that in *S. cerevisiae*, Cdc14p may activate the MEN component Cdc15p providing the potential for a positive feedback loop to promote exit from mitosis (Jaspersen and Morgan, 2000). Whether this is the case in *S. pombe* will be addressed in future studies.

Septation is partially inhibited in the *flp1::kanMX6 cdc16-116* mutant at 25°C. The single *cdc16-116* mutant does not show any obvious septation defect at 25°C (Minet et al., 1979). However, increased expression of the cloned *cdc16-116* allele in wild-type cells blocks septum formation, while *cdc16+* does not (L. Cerutti and V.S., unpublished), suggesting that the mutant cdc16-116p may be a more effective negative regulator of SIN signalling. If the absence of flp1p also attenuates SIN signalling, a combination of these mutants may produce a synergistic effect. Growth at a higher temperature, such as 29°C, may partially inactivate cdc16-116p, reducing the inhibitory effect.

Previous studies have demonstrated that cdc2p kinase activity during mitosis is antagonistic to septum formation (Cerutti and Simanis, 1999; He et al., 1998; He et al., 1997). Thus, premature activation of cdc2p, and its delayed or incomplete inactivation during mitosis might account for the strong negative genetic interactions of *flp1::kanMX6* with mutants that have reduced SIN signalling, and the greatly enhanced septation defects shown by *flp1::kanMX6* in *wee1−* and *cdc2w* backgrounds.

What is the role of flp1p in the morphology checkpoint?

The involvement of flp1p in the *S. pombe* morphology checkpoint could be at several levels. One possibility is that it is an essential effector of the checkpoint, and that its activation (or release from the nucleolus) is required to delay the next cycle of nuclear division, and the resumption of tip-growth. Alternatively, since the morphology checkpoint requires both wee1p and SIN signalling for its activity, it is possible that the failure of the checkpoint results from reduced SIN signalling in the *flp1::kanMX6* cells.

What are the physiological substrates of flp1p?

Our studies suggest that elements of the mitotic control system (or their upstream regulators) may be targets of flp1p. Flp1p is also located on the spindle pole body, mitotic spindle and the contractile ring. It is known that many regulators of mitosis and septum formation, are located on the spindle pole body (Alfa et al., 1990; Bahler et al., 1998a; Cerutti and Simanis, 1999; Chang and Gould, 2000; Eng et al., 1998; Guertin et al., 2000; Hou et al., 2000; Mulvihill et al., 1999; Sohrmann et al., 1998; Sparks et al., 1999), so these may be among the targets of flp1p. The diversity of phenotypes associated with loss of flp1p function may reflect the fact that it has more than one important substrate, and so its absence may reduce the fidelity, or efficiency, of several processes. Identification of the substrates and anchors of flp1p on the mitotic spindle, the contractile ring and the nucleolus will be the subject of future studies.

REFERENCES

Alfa, C. E., Ducommun, B., Beach, D. and Hyams, J. S. (1990). Distinct nuclear and spindle pole body population of cyclin-cdc2 in fission yeast. *Nature* **347**, 680-682.

Aligue, R., Wu, L. and Russell, P. (1997). Regulation of Schizosaccharomyces pombe Wee1 tyrosine kinase. *J. Biol. Chem.* **272**, 13320-13325.

Aris, J. P. and Blobel, G. (1991). cDNA cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. *Proc. Natl. Acad. Sci. USA* **88**, 931-935.

Bahler, J., Steever, A. B., Wheatley, S., Wang, Y., Pringle, J. R., Gould, K. L. and McCollum, D. (1998a). Role of polo kinase and Mid1p in determining the site of cell division in fission yeast. *J. Cell Biol.* **143**, 1603-1616.

Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., III, Steever, A. B., Wach, A., Philippsen, P. and Pringle, J. R. (1998b). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast* **14**, 943-951.

Balasubramanian, M. K., McCollum, D., Chang, L., Wong, K. C., Naqvi, N. I., He, X., Sazer, S. and Gould, K. L. (1998). Isolation and characterization of new fission yeast cytokinesis mutants. *Genetics* **149**, 1265-1275.

Balasubramanian, M. K., McCollum, D. and Gould, K. L. (1997). Cytokinesis in fission yeast Schizosaccharomyces pombe. *Methods Enzymol.* **283**, 494-506.

Balasubramanian, M. K., McCollum, D. and Surana, U. (2000). Tying the knot: linking cytokinesis to the nuclear cycle. *J. Cell Sci.* **113**, 1503-1513.

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.

Basi, G., Schmid, E. and Maundrell, K. (1993). TATA box mutations in the Schizosaccharomyces pombe nmt1 promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. *Gene* **123**, 131-136.

Blanco, M. A., Sanchez-Diaz, A., de Prada, J. M. and Moreno, S. (2000). APC(ste9/srw1) promotes degradation of mitotic cyclins in G(1) and is inhibited by cdc2 phosphorylation. *EMBO J.* **19**, 3945-3955.

Breeding, C. S., Hudson, J., Balasubramanian, M. K., Hemmingsen, S. M., Young, P. G. and Gould, K. L. (1998). The cdr2(+) gene encodes a regulator of G2/M progression and cytokinesis in Schizosaccharomyces pombe. *Mol. Biol. Cell* **9**, 3399-3415.

Cerutti, L. and Simanis, V. (1999). Asymmetry of the spindle pole bodies and spg1p GAP segregation during mitosis in fission yeast. *J. Cell Sci.* **112**, 2313-2321.

Chang, L. and Gould, K. L. (2000). Sid4p is required to localize components of the septation initiation pathway to the spindle pole body in fission yeast. *Proc. Natl. Acad. Sci. USA* **97**, 5249-5254.

Creanor, J. and Mitchison, J. M. (1996). The kinetics of the B cyclin p56cdc13 and the phosphatase p80cdc25 during the cell cycle of the fission yeast Schizosaccharomyces pombe. *J. Cell Sci.* **109**, 1647-1653.

Ducommun, B., Draetta, G., Young, P. and Beach, D. (1990). Fission yeast cdc25 is a cell-cycle regulated protein. *Biochem. Biophys. Res. Commun.* **167**, 301-309.

Eng, K., Naqvi, N. I., Wong, K. C. and Balasubramanian, M. K. (1998). Rng2p, a protein required for cytokinesis in fission yeast, is a component of the actomyosin ring and the spindle pole body. *Curr. Biol.* **8**, 611-621.

Enoch, T. and Nurse, P. (1990). Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell* **60**, 665-673.

Fankhauser, C., Marks, J., Reymond, A. and Simanis, V. (1993). The S. pombe cdc16 gene is required both for maintenance of p34cdc2 kinase activity and regulation of septum formation: a link between mitosis and cytokinesis? *EMBO J.* **12**, 2697-2704.

Fankhauser, C., Reymond, A., Cerutti, L., Utzig, S., Hofmann, K. and Simanis, V. (1995). The S. pombe cdc15 gene is a key element in the reorganization of F- actin at mitosis. *Cell* **82**, 435-444.

Fankhauser, C. and Simanis, V. (1993). The Schizosaccharomyces pombe cdc14 gene is required for septum formation and can also inhibit nuclear division. *Mol. Biol. Cell* **4**, 531-539.

Fankhauser, C. and Simanis, V. (1994). The cdc7 protein kinase is a dosage dependent regulator of septum formation in fission yeast. *EMBO J.* **13**, 3011-3019.

Fantes, P. A. (1981). Isolation of cell size mutants of a fission yeast by a new selective method: characterization of mutants and implications for division control mechanisms. *J. Bacteriol.* **146**, 746-754.

Fantes, P. A. (1983). Control of timing of cell cycle events in fission yeast by the wee 1+ gene. *Nature* **302**, 153-155.

Fitzpatrick, P. J., Toyn, J. H., Millar, J. B. and Johnston, L. H. (1998). DNA replication is completed in Saccharomyces cerevisiae cells that lack functional Cdc14, a dual-specificity protein phosphatase. *Mol. Gen. Genet.* **258**, 437-441.

Fournier, N., Cerutti, L., Beltraminelli, N., Salimova, E. and Simanis, V. (2001). Bypass of the requirement for cdc16p GAP function in Schizosaccharomyces pombe by mutation of the septation initiation network genes. *Arch. Microbiol.* **175**, 62-69.

Furge, K. A., Wong, K., Armstrong, J., Balasubramanian, M. and Albright, C. F. (1998). Byr4 and Cdc16 form a two-component GTPase-activating protein for the Spg1 GTPase that controls septation in fission yeast. *Curr. Biol.* **8**, 947-954.

Guertin, D. A., Chang, L., Irshad, F., Gould, K. L. and McCollum, D. (2000). The role of the sid1p kinase and cdc14p in regulating the onset of cytokinesis in fission yeast. *EMBO J.* **19**, 1803-1815.

Hagan, I. and Yanagida, M. (1995). The product of the spindle formation gene sad1+ associates with the fission yeast spindle pole body and is essential for viability. *J. Cell Biol.* **129**, 1033-1047.

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.

He, X., Jones, M. H., Winey, M. and Sazer, S. (1998). Mph1, a member of the Mps1-like family of dual specificity protein kinases, is required for the spindle checkpoint in S. pombe. *J. Cell Sci.* **111**, 1635-1647.

He, X., Patterson, T. E. and Sazer, S. (1997). The Schizosaccharomyces pombe spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. *Proc. Natl. Acad. Sci. USA* **94**, 7965-7970.

Henriquez, R., Blobel, G. and Aris, J. P. (1990). Isolation and sequencing of NOP1. A yeast gene encoding a nucleolar protein homologous to a human autoimmune antigen. *J. Biol. Chem.* **265**, 2209-2215.

Hirano, T., Konoha, G., Toda, T. and Yanagida, M. (1989). Essential roles of the RNA polymerase I largest subunit and DNA topoisomerases in the formation of fission yeast nucleolus. *J. Cell Biol.* **108**, 243-253.

Hou, M. C., Salek, J. and McCollum, D. (2000). Mob1p interacts with the sid2p kinase and is required for cytokinesis in fission yeast. *Curr. Biol.* **10**, 619-622.

Hoyt, M. A. (2000). Exit from mitosis: spindle pole power. *Cell* **102**, 267-270.

Jaspersen, S. L., Charles, J. F. and Morgan, D. O. (1999). Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14. *Curr. Biol.* **9**, 227-236.

Jaspersen, S. L., Charles, J. F., Tinker-Kulberg, R. L. and Morgan, D. O. (1998). A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae. *Mol. Biol. Cell* **9**, 2803-2817.

Jaspersen, S. L. and Morgan, D. O. (2000). Cdc14 activates cdc15 to promote mitotic exit in budding yeast. *Curr. Biol.* **10**, 615-618.

Kanoh, J. and Russell, P. (1998). The protein kinase Cdr2, related to Nim1/Cdr1 mitotic inducer, regulates the onset of mitosis in fission yeast. *Mol. Biol. Cell* **9**, 3321-3334.

Kitamura, K., Maekawa, H. and Shimoda, C. (1998). Fission yeast Ste9, a homolog of Hct1/Cdh1 and Fizzy-related, is a novel negative regulator of cell cycle progression during G1-phase. *Mol. Biol. Cell* **9**, 1065-1080.

Le Goff, X., Utzig, S. and Simanis, V. (1999a). Controlling septation in fission yeast: finding the middle, and timing it right. *Curr. Genet.* **35**, 571-584.

Le Goff, X., Woollard, A. and Simanis, V. (1999b). Analysis of the cps1 gene provides evidence for a septation checkpoint in Schizosaccharomyces pombe. *Mol. Gen. Genet.* **262**, 163-172.

Li, L., Ernsting, B. R., Wishart, M. J., Lohse, D. L. and Dixon, J. E. (1997). A family of putative tumor suppressors is structurally and functionally conserved in humans and yeast. *J. Biol. Chem.* **272**, 29403-29406.

Liu, J., Wang, H. and Balasubramanian, M. K. (2000). A checkpoint that monitors cytokinesis in schizosaccharomyces pombe. *J. Cell Sci.* **113**, 1223-1230.

Marks, J., Hagan, I. M. and Hyams, J. S. (1986). Growth polarity and cytokinesis in fission yeast: the role of the cytoskeleton. *J. Cell. Sci. Suppl.* **5**, 229-241.

Minet, M., Nurse, P., Thuriaux, P. and Mitchison, J. M. (1979). Uncontrolled septation in a cell division cycle mutant of the fission yeast Schizosaccharomyces pombe. *J. Bacteriol.* **137**, 440-446.

Moreno, S., Hayles, J. and Nurse, P. (1989). Regulation of p34cdc2 protein kinase during mitosis. *Cell* **58**, 361-372.

Moreno, S., Klar, A. and Nurse, P. (1991). Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. *Methods Enzymol.* **194**, 795-823.

Moreno, S., Nurse, P. and Russell, P. (1990). Regulation of mitosis by cyclic accumulation of p80cdc25 mitotic inducer in fission yeast. *Nature* **344**, 549-552.

Mulvihill, D. P., Petersen, J., Ohkura, H., Glover, D. M. and Hagan, I. M. (1999). Plo1 kinase recruitment to the spindle pole body and its role in cell division in Schizosaccharomyces pombe. *Mol. Biol. Cell.* **10**, 2771-2785.

Norbury, C. and Moreno, S. (1997). Cloning cell cycle regulatory genes by transcomplementation in yeast. *Methods Enzymol.* **283**, 44-59.

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.

Ohkura, H., Hagan, I. M. and Glover, D. M. (1995). The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. *Genes Dev.* **9**, 1059-1073.

Potashkin, J. A., Derby, R. J. and Spector, D. L. (1990). Differential distribution of factors involved in pre-mRNA processing in the yeast cell nucleus. *Mol. Cell Biol.* **10**, 3524-3534.

Russell, P. and Nurse, P. (1987a). The mitotic inducer nim1+ functions in a

regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell* **49**, 569-576.

Russell, P. and Nurse, P. (1987b). Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. *Cell* **49**, 559-567.

Salimova, E., Sohrmann, M., Fournier, N. and Simanis, V. (2000). The S. pombe orthologue of the S. cerevisiae mob1 gene is essential and functions in signalling the onset of septum formation. *J. Cell Sci.* **113**, 1695-1704.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning*. New York: Cold Spring Harbor Laborator Press.

Sawin, K. E. (2000). Cytokinesis: Sid signals septation. *Curr. Biol.* **10**, R547-R550.

Schmidt, S., Sohrmann, M., Hofmann, K., Woollard, A. and Simanis, V. (1997). The Spg1p GTPase is an essential, dosage-dependent inducer of septum formation in Schizosaccharomyces pombe. *Genes Dev.* **11**, 1519-1534.

Shirayama, M., Toth, A., Galova, M. and Nasmyth, K. (1999). APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature* **402**, 203-207.

Shou, W., Seol, J. H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z. W., Jang, J., Charbonneau, H. and Deshaies, R. J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* **97**, 233-244.

Sohrmann, M., Fankhauser, C., Brodbeck, C. and Simanis, V. (1996). The dmf1/mid1 gene is essential for correct positioning of the division septum in fission yeast. *Genes Dev* **10**, 2707-2719.

Sohrmann, M., Schmidt, S., Hagan, I. and Simanis, V. (1998). Asymmetric segregation on spindle poles of the Schizosaccharomyces pombe septum-inducing protein kinase Cdc7p. *Genes Dev.* **12**, 84-94.

Song, K., Mach, K. E., Chen, C. Y., Reynolds, T. and Albright, C. F. (1996). A novel suppressor of ras1 in fission yeast, byr4, is a dosage- dependent inhibitor of cytokinesis. *J. Cell Biol.* **133**, 1307-1319.

Sparks, C. A., Morphew, M. and McCollum, D. (1999). Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. *J. Cell Biol.* **146**, 777-790.

Straight, A. F., Shou, W., Dowd, G. J., Turck, C. W., Deshaies, R. J., Johnson, A. D. and Moazed, D. (1999). Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell* **97**, 245-256.

Sveiczer, A., Novak, B. and Mitchison, J. M. (1999). Mitotic control in the absence of cdc25 mitotic inducer in fission yeast. *J. Cell Sci.* **112**, 1085-1092.

Taylor, G. S., Liu, Y., Baskerville, C. and Charbonneau, H. (1997). The activity of Cdc14p, an oligomeric dual specificity protein phosphatase from Saccharomyces cerevisiae, is required for cell cycle progression. *J. Biol. Chem.* **272**, 24054-24063.

Visintin, R., Craig, K., Hwang, E. S., Prinz, S., Tyers, M. and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol. Cell* **2**, 709-718.

Visintin, R., Hwang, E. S. and Amon, A. (1999). Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature* **398**, 818-823.

Wan, J., Xu, H. and Grunstein, M. (1992). CDC14 of Saccharomyces cerevisiae. Cloning, sequence analysis, and transcription during the cell cycle. *J. Biol. Chem.* **267**, 11274-11280.

Weilguny, D., Praetorius, M., Carr, A., Egel, R. and Nielsen, O. (1991). New vectors in fission yeast: application for cloning the his2 gene. *Gene* **99**, 47-54.

Wu, L. and Russell, P. (1993). Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. *Nature* **363**, 738-741.

Wu, L. and Russell, P. (1997). Nif1, a novel mitotic inhibitor in Schizosaccharomyces pombe. *EMBO J.* **16**, 1342-1350.

Wu, L., Shiozaki, K., Aligue, R. and Russell, P. (1996). Spatial organization of the Nim1-Wee1-Cdc2 mitotic control network in Schizosaccharomyces pombe. *Mol. Biol. Cell* **7**, 1749-1758.

Yamaguchi, S., Murakami, H. and Okayama, H. (1997). A WD repeat protein controls the cell cycle and differentiation by negatively regulating Cdc2/B-type cyclin complexes. *Mol. Biol. Cell* **8**, 2475-2486.

Yamaguchi, S., Okayama, H. and Nurse, P. (2000). Fission yeast Fizzy-related protein srw1p is a G(1)-specific promoter of mitotic cyclin B degradation. *EMBO J.* **19**, 3968-3977.

Zeng, Y. and Piwnica-Worms, H. (1999). DNA damage and replication checkpoints in fission yeast require nuclear exclusion of the Cdc25 phosphatase via 14-3-3 binding. *Mol. Cell Biol.* **19**, 7410-7419.