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Requirement for PP1 phosphatase and 20S cyclosome/APC for the onset of anaphase is lessened by the dosage increase of a novel gene *sds23*$^+$

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Ubiquitin-dependent proteolysis is required for the onset of anaphase. We show that protein dephosphorylation by protein phosphatase 1 (PP1) is also essential for initiating anaphase in fission yeast. PP1 may directly or indirectly regulate the 20S cyclosome/APC (anaphase-promoting complex) required for anaphase-promoting proteolysis. Using anti-phosphopeptide antibodies, PP1 is shown to be dephosphorylated at the C-terminus, upon the onset of anaphase, for reactivation. *sds23*$^+$, a novel gene, is a multicopy suppressor for mutations in PP1 and the 20S cyclosome/APC, implying that the gene dosage increase can relieve the requirement for PP1 and the cyclosome/APC for the onset of anaphase. The *sds23*$^+$ gene is not essential for cell viability, but a mutant with the gene deleted cannot form colonies at 22 and 36°C. In the *sds23* deletion mutant, the progression of anaphase and cytokinesis is retarded and cell shape is aberrant. These defects are overcome by plasmids carrying the genes encoding subunits of the 20S cyclosome/APC or PP1. These results demonstrate functions other than promoting anaphase for the components of the 20S cyclosome/APC and also a close functional relationship of Sds23 with PP1 and 20S cyclosome/APC.

Keywords: cyclosome-APC/mitosis/proteasome/20S E3 ubiquitin ligase/*Schizosaccharomyces pombe*

Introduction

A genetic approach in fission yeast has identified several essential genes required for the progression from metaphase to anaphase (reviewed in Yanagida, 1989, 1995). Mutations in *nuc2*$^+$ and *cut9*$^+$ genes (Hirano *et al.*, 1988; Samejima and Yanagida, 1994) lead to an arrest in metaphase, and those genes encode proteins containing a repeat motif called the TPR (trichopeptide repeat; Sikorski *et al.*, 1990; reviewed in Goebl and Yanagida, 1991). The TPR motifs are perhaps required for intra- and/or intermolecular protein interactions (Hirano *et al.*, 1990; Lamb *et al.*, 1994), but the actual functions of the TPR gene products are not well understood. Nuc2 and Cut9 proteins are similar to the *Saccharomyces cerevisiae* Cdc27 and Cdc16, respectively, and their human homologs (Tugendreich *et al.*, 1995). They have been shown recently to be the components of the 20S complex with E3 ubiquitin ligase activity essential for the proteolysis of cyclin B (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). This 20S complex is called the cyclosome (Sudakin *et al.*, 1995) or APC, anaphase-promoting complex (King *et al.*, 1995). Fission yeast Nuc2 and Cut9 proteins are also shown to be essential components of the 20S cyclosome (H.Yamada, K.Kumada and M.Yanagida, manuscript in preparation).

The essential role of ubiquitin-dependent proteolysis in the progression from metaphase to anaphase was supported further by the metaphase arrest phenotypes of mutants defective in the subunits of 26S proteasome (Ghislain *et al.*, 1993; Gordon *et al.*, 1993). Ubiquitin-dependent proteolysis involves the destruction of cyclin B (Glotzer *et al.*, 1991; Hershko *et al.*, 1991, 1994). Furthermore, ubiquitin-dependent proteolysis is also required for sister chromatid separation in anaphase (Holloway *et al.*, 1993; Surana *et al.*, 1993; Irniger *et al.*, 1995; Funabiki *et al.*, 1996). Components other than cyclin B have to be degraded for the separation of sister chromatids. A fission yeast protein Cut2 is degraded periodically in anaphase and this destruction requires Cut9, a component of the 20S complex (Funabiki *et al.*, 1996). Cut2 contains a sequence essential for this periodic destruction, and the sequence can be substituted by that of cyclin B to complement a temperature-sensitive (ts) *cut2* mutation.

Although the requirement for proteolysis in anaphase is fairly well documented, the role of protein dephosphorylation by type 1 phosphatase (PP1) in anaphase is not clear. Mutations in PP1 impair the exit from mitosis in filamentous fungi (Doonan and Morris, 1989), *Schizosaccharomyces pombe* (Ohkura *et al.*, 1989) and *Drosophila* (Axton *et al.*, 1990). Fission yeast have two type 1 protein phosphatases, Dis2 and Sds21 (Ohkura *et al.*, 1989). Their amino acid sequences are nearly 80% identical to each other and to the mammalian PP1 homologs. Single gene disruption mutants Δ*dis2* or Δ*sds21* are viable, but the double deletion mutant Δ*dis2* Δ*sds21* is non-viable, indicating that PP1 is essential for viability and that *dis2*$^+$ and *sds21*$^+$ are functionally redundant. A cold-sensitive (cs) mutant *dis2-11* was isolated by its characteristic *dis* phenotype at the restrictive temperature, 20°C (Ohkura *et al.*, 1988): the spindle elongates in the absence of sister chromatid separation, while the H1 kinase activity remains high (Ohkura *et al.*, 1988; Kinoshita *et al.*, 1991). This *dis2-11* allele is dominant in haploid cells (the genomic *sds21*$^+$ did not suppress *dis2-11*; Ohkura *et al.*, 1989; Kinoshita *et al.*, 1990). However, in heterozygous diploid cells, *dis2-11* was a recessive mutation.

The *sds22*$^+$ gene was isolated as a multicopy suppressor of *dis2-11* (Ohkura and Yanagida, 1991). Sds22 protein containing a leucine-rich repeat physically interacts with the catalytic subunits of Dis2 and Sds21 phosphatase

(Stone *et al.*, 1993). Sds22 is evolutionarily conserved (Wilson *et al.*, 1994; Hisamoto *et al.*, 1995; MacKelvie *et al.*, 1995; Renouf *et al.*, 1995). In the absence of Sds22 protein, cells were blocked in metaphase (Ohkura and Yanagida, 1991). A ts *sds22* mutant with the N-terminal portion deleted also displayed the metaphase arrest phenotype (Stone *et al.*, 1993): mutant cells failed in sister chromatid separation, whereas the spindle was formed with condensed chromosomes and high H1 kinase activity. As the Sds22–Dis2 and Sds22–Sds21 complexes exhibit specific phosphatase activity, we proposed that Sds22 acts as a mitotic regulator of PP1 and is essential for the progression from metaphase to anaphase (Stone *et al.*, 1993). The major target substrates for PP1 in mitosis, however, remain to be determined.

Dis2 and Sds21 phosphatases are functionally not identical. It was reported previously that a *sds22* deletion mutant was rescued by pSDS21 but not by pDIS2 (Ohkura and Yanagida, 1991; Stone *et al.*, 1993). We reasoned that Dis2 might be down-regulated when overexpressed but Sds21 might not be (Yamano *et al.*, 1994). Such down-regulation could be realized by a phosphorylation event: Sds21 was hardly phosphorylated, whereas Dis2 activity was inhibited by phosphorylation at the C-terminal Cdc2 site (the 316th threonine).

To understand the role of PP1 in exiting mitosis, we have been trying to identify proteins which may interact with either the catalytic subunits or their regulatory proteins such as Sds22. One approach was to isolate genes which could suppress the mutant phenotypes when the gene dosage was elevated, using a multicopy plasmid or an inducible promoter. Another approach was to identify extragenic suppressor mutations that suppressed the PP1 mutations. A third and more direct approach is the two-hybrid screen using the catalytic or regulatory units as bait.

Here we report the isolation of a high copy suppressor (designated *sds23*$^+$) for the ts *sds22* mutation. The *sds23*$^+$ gene also suppressed the cs phenotype of *dis2-11*. Surprisingly, the same multicopy plasmid suppressed ts *nuc2* and *cut9* mutations as well. This is the first evidence that ubiquitin-dependent proteolysis is functionally linked to PP1 protein phosphatase. We will discuss potential roles of Sds23 and the relationship of PP1 with the 20S cyclosome/APC and/or Sds23 in the progression from metaphase to anaphase.

Results

Detection of phosphorylated T316 by antibodies

Rabbit polyclonal antibodies were prepared against a phosphopeptide containing phosphorylated T316 of the Dis2 catalytic subunit (hereafter called T316P; Figure 1A) and were employed to monitor the degree of phosphorylation at the T316 site in cell extracts by immunoblotting (Figure 1B). T316P antibodies (αT316P) produced a very faint band in growing *S.pombe* cells (wt in left panel of Figure 1B), but not in dis2-deleted cells (Δ*dis2* in left panel). The immunoblot pattern obtained by polyclonal antibodies against Dis2 (αD2F) is shown as a control (right panel).

Wild-type Dis2 and mutant Dis2 with the T residue at position 316 substituted with A were overproduced under the control of the inducible promoter *nmt1* (REP1, Maundrell, 1990). Overexpression of these gene products occurred 10 h after the removal of thiamine. An intense band was seen for wild-type Dis2 using anti-T316P antibodies (Dis2, left panel in Figure 1C). In contrast, no band was detected when mutant T316A protein was overproduced (Dis2T316A in left panel). This indicated that anti-T316P antibodies specifically recognize the phosphorylated form of Dis2. Anti-D2F antibodies, which recognized both phosphorylated and non-phosphorylated Dis2, detected bands for both wild-type and mutant proteins (right panel).

These results are consistent with the colony-forming ability of cells overproducing Dis2 under a strong promoter. The activity of the Dis2 produced was perhaps inhibited by phosphorylation at T316 (Figure 1D, –Thi, Dis2), which allows for colony formation. Cells producing the mutant T316A protein under the strong promoter, on the other hand, severely blocked colony formation (Figure 1D, –Thi, Dis2T316A), consistent with the previous finding that mutant T316A retained phosphatase activity (Yamano *et al.*, 1994). Overproduced Sds21 which lacks the phosphorylation site also inhibited colony formation (Yamano *et al.*, 1994).

Inhibition of Dis2 by cell cycle-dependent phosphorylation at T316

The above results indicated that T316 of Dis2 was phosphorylated when there was an excess of Dis2. It was shown that T316 of Dis2 was hyperphosphorylated in metaphase-arrested cells and that the Dis2 protein phosphorylated at this site contained a significantly reduced phosphatase activity (Yamano *et al.*, 1994). We then examined whether T316 phosphorylation occurs in normal mitosis. A temperature shift (G_2 block and release) experiment using the *cdc25-22* mutant showed that the level of T316-phosphorylated Dis2 peaked in mitosis (Figure 2A). G2-arrested *cdc25* cells at 36°C were released at 26°C. Cell extracts were then prepared at certain intervals, and the band intensity of phosphorylated Dis2 at T316 was examined by anti-T316P antibodies. Immunoblotting using anti-dis2 (D2F), anti-cyclin B (Cdc13) and anti-cdc2 (PSTAIR) antibodies was also carried out as a control.

The band intensity detected by anti-T316P peaked ~30 min after the release (top panel of Figure 2A), in parallel with the peak of H1 kinase activity (right panel); however, the protein level of Dis2 did not fluctuate (αD2F). The level of cyclin B (Cdc13) and H1 kinase activity decreased at 40–50 min (third panel), which corresponded to the decrease in the level of the T316P band. These results indicated that phosphorylation at T316 is regulated during normal mitosis. The decrease in T316 phosphorylation occurred simultaneously with the cyclin destruction. This then led to the activation of Dis2 phosphatase.

The metaphase-arrested ts *nuc2-663* cells cultured at 36°C for 4 h showed an intense band with anti-T316P antibodies (left panel, Figure 2B), while the band intensity was weak in the G_2-arrested *cdc25* mutant. Similarly to those of the *nuc2* mutant, cs *nda3-311* mutant cells which were cultured at 20°C for 8 h and arrested at a prophase-like stage also showed an intense T316P band (right panel). At the permissive temperature (33°C) the band intensity was weak. These results suggested that T316 phosphorylation occurs when the mitotic kinase is high.

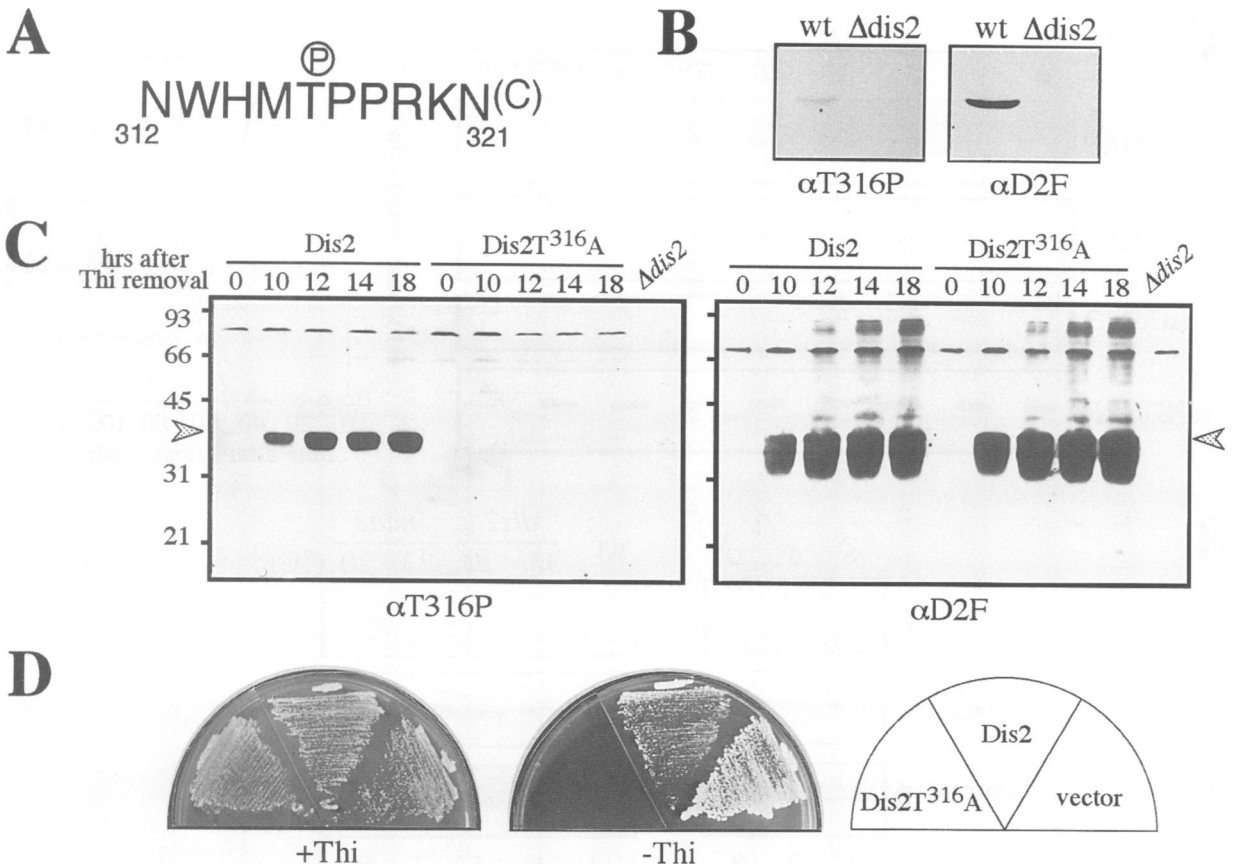

Fig. 1. Detection of phosphorylated T316 by anti-phosphopeptide antibodies. (**A**) A phosphopeptide used for raising rabbit antibodies. (**B**) Immunoblot of wild-type and Δ*dis2* cells using anti-phosphopeptide (αT316P) and anti-dis2 antibodies (αD2F; Stone *et al.*, 1993). (**C**) Immunoblotting of wild-type cells carrying plasmid nmt-Dis2 or nmt-Dis2T316A under the nmt (REP1) promoter using anti-T316P antibodies (left panel) and anti-dis2 antibodies (right panel). Wild-type Dis2 or mutant Dis2T316A was produced after 10 h in the absence of thiamine. Anti-T316P antibodies did not recognize the mutant Dis2T316A, while anti-dis2 antibodies detected both wild-type and mutant proteins. (**D**) Wild-type cells carrying plasmid nmt-Dis2T316A, nmt-Dis2 or vector were plated in the presence (+Thi, the promoter off) or the absence of thiamine (–Thi, the promoter on) at 36°C. No colony was made for wild-type cells carrying plasmid nmt-Dis2T316A.

Thus there are three conditions under which a high level of T316 phosphorylation occurs: (i) an excess of Dis2, (ii) normal mitosis and (iii) metaphase-arrested cells. In the cs *dis2-11* mutant, weak phosphorylation occurred at both 20 and 33°C.

Deletion of the PP1 genes leads to metaphase arrest

Mutations in *nuc2-663*, *cut9-665* and *sds22-181* have been shown to be defective in metaphase/anaphase progression (Hirano *et al.*, 1988; Ohkura and Yanagida, 1991; Stone *et al.*, 1993; Samejima and Yanagida, 1994). However, *dis2-11* displayed an allele-specific mitotic phenotype with spindle elongation (Ohkura *et al.*, 1989; see Figure 3A). To understand precisely the mitotic function of PP1, it was essential to determine the phenotype of the double deletion mutant Δ*dis2* Δ*sds21*.

Spores deleted for both *dis2*$^+$ and *sds21*$^+$ genes were made by crossing, and were germinated on an agar plate (Materials and methods). Each of the double deletion segregants Δ*dis2* Δ*sds21* remained as single cells after germination at 30°C for 20 h. Such germinated single cells were stained on the plate with a fluorescent probe, 4′,6-diaminino-2-phenylindole (DAPI), and observed under an epifluorescence microscope. More than 90% of the cells examined contained condensed chromosomes (Figure 3B, upper panel). These were highly similar to the metaphase-arrested cells seen in *sds22* mutant cells (Figure 3B, middle right) cultured at the restrictive temperature (Ohkura and Yanagida, 1991; Stone *et al.*, 1993). Wild-type and arrested *dis2-11* cells are also shown as control (Figure 3B, bottom left and right, respectively).

To determine whether Δ*dis2* Δ*sds21* cells contained the mitotic spindle, anti-tubulin (TAT1) and anti-sad1 antibodies were applied to immunolocalize microtubules and spindle pole bodies (SPBs), respectively, in the double gene-disrupted cells (Woods *et al.*, 1989; Hagan and Yanagida, 1995). For this purpose, Δ*dis2* Δ*sds21* cells carrying the plasmid pDIS2 were grown under non-selective conditions, to facilitate plasmid loss. In parallel with the increase in plasmid loss, cells containing condensed chromosomes with a short metaphase spindle dramatically increased (Figure 3C). After transferring the culture from selective to non-selective medium at 33°C, the fraction of these metaphase-arrested cells increased about nine times within 20 h (from 2 to 19%). We concluded, therefore, that the complete absence of PP1 in fission yeast leads to the metaphase arrest, which is strikingly similar to the phenotype of *sds22* mutants. The *dis2-11* mutation is allele-specific and semi-dominant. Its

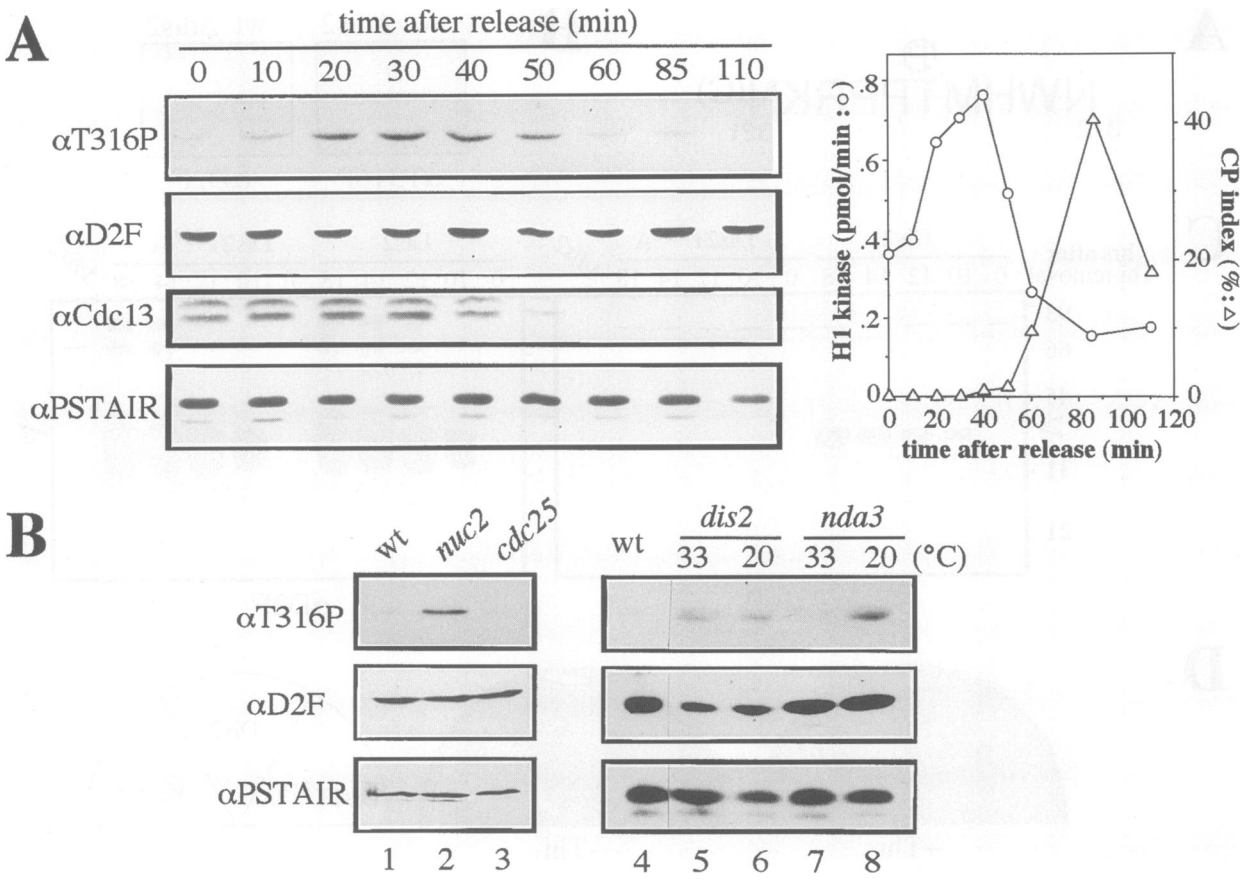

Fig. 2. Cell cycle-dependent phosphorylation at the C-terminal T316 site. (**A**) Left panel: synchronous culture analysis of phosphorylation at the T316 residue of Dis2 using anti-T316P antibodies. The culture of the ts *cdc25* mutant was arrested in late G_2 at 36°C and released at 26°C. Then, cells were taken and extracts were immunoblotted using anti-T316P, anti-dis2 (D2F), anti-cyclin B (Cdc13) and anti-cdc2 (PSTAIR) antibodies. Right panel: the level of T316P peaked when the H1 kinase activity (○) was also maximal. The percentage septation index (CP index) was also measured (△), which peaked at 85 min. (**B**) Left panel: extracts of wild-type (wt), *nuc2-663* and *cdc25* mutants were run in SDS–PAGE and immunoblotted using anti-T316P, anti-dis2 and anti-cdc2 antibodies. Only *nuc2-663* extracts displayed the band by anti-T316P antibodies. Right panel: extracts of wild-type, *dis2-11* and *nda3-311* mutants were also immunoblotted using the same antibodies. These cs mutants were grown initially at 33°C and then transferred to 20°C for 8 h.

phenotype is also distinct. The *dis* phenotype observed in *dis2-11* at the restrictive temperature was not found in either Δ*dis2* or Δ*dis2* Δ*sds21*. The *dis2-11* phenotype can be considered as a 'leaky' anaphase phenotype, because the spindle elongates in the absence of sister chromatid separation. Restraining of spindle extension until anaphase might be disrupted in *dis2-11* as well as in *dis1* mutant cells (Nabeshima *et al.*, 1995). The above results established that PP1 phosphatase is required for the progression from metaphase to anaphase and that PP1 activity is down-regulated at metaphase in fission yeast.

Isolation of sds23$^+$ as a multicopy suppressor for PP1 mutants

A multicopy plasmid which conferred the ability to suppress ts *sds22-181* was isolated. Briefly, *sds22-181* was transformed by plasmids using a genomic DNA library of *S.pombe* at 26°C, and the resulting transformants were replica plated and incubated at 33°C (Materials and methods). Twenty seven plasmids conferring growth of *sds22-181* at 33°C were recovered by screening >40 000 transformants. By hybridization and partial nucleotide sequencing, all plasmids but one (pEP200) were either carrying *sds21$^+$* or *sds22$^+$*. A subcloned plasmid pEP205 (Figure 4B; designated pSDS23) suppressed the ts phenotype of *sds22-181* at 33°C but not at 36°C (Figure 4A, upper panel). Control plasmids pSDS21 and pSDS22 could suppress *sds22-181* at 36°C as well (Ohkura and Yanagida, 1991). pSDS23 also suppressed the cs phenotype of the *dis2-11* mutant (Figure 4A, lower panel) at 22°C. The suppression ability of pSDS23 for *dis2-11* was comparable with that of pDIS2, pSDS21 and pSDS22.

The minimal fragment for suppression is a 3.2 kb *Pvu*II–*Sac*I fragment in the insert of pEP205 (Figure 4B). This fragment was sequenced (Figure 4C) and was found to contain only one open reading frame, which was designated the *sds23$^+$* gene (see Materials and methods). Hybridization of an ordered cosmid bank using pSDS23 as the probe located *sds23$^+$* at a locus between *cut3$^+$* and *dsk1$^+$* in chromosome II (Mizukami *et al.*, 1993).

The *sds23$^+$* gene encodes a predicted gene product of 408 amino acids and appears to be conserved. The genome of the budding yeast *S.cerevisiae* reveals two open reading frames (YBR214w and YGL056c), both of which are 40% identical to Sds23 (Figure 4D). No other protein in the available databases is significantly similar to Sds23.

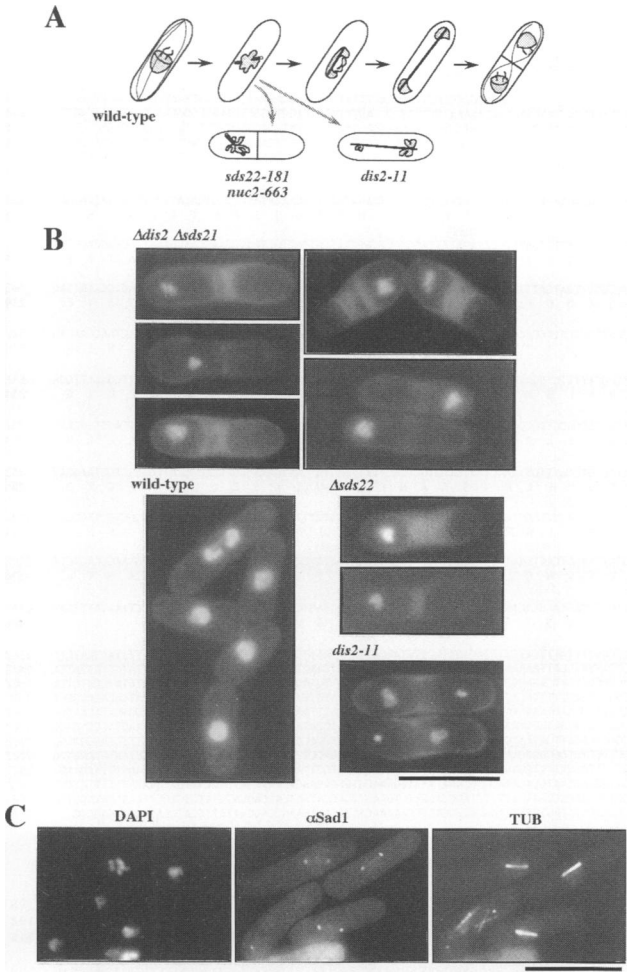

Fig. 3. Phenotype of the double mutant Δ*dis2* Δ*sds21*. (**A**) Wild-type mitosis that illustrates nuclear division and spindle dynamics. The half tone represents the nucleus, while thin and thick lines show cytoplasmic and spindle microtubules, respectively. Mutant phenotypes are also schematized. (**B**) Germinated cells from the double mutant Δ*dis2* Δ*sds21*, single deletion mutants Δ*sds22*, wild-type and cs *dis2-11* were stained with DAPI. The bar indicates 10 μm. (**C**) The double mutant Δ*dis2* Δ*sds21*cells carrying pDIS2 were grown in the selective medium and then transferred to the rich medium and incubated at 33°C for 20 h. The number of cells which lost plasmid increased. Cells were stained with anti-sad1, anti-tubulin (TUB) antibodies and DAPI. Cells containing the short metaphase spindle and condensed chromosomes were frequently seen. The bar indicates 10 μm.

Suppression of nuc2 and cut9 mutations by the multicopy sds23$^+$ gene

In an independent screening, we isolated a high gene dosage plasmid (pKK2) which partly suppressed the ts phenotype of the *nuc2-663* mutant: mutant cells carrying pKK2 produced colonies at 30°C. Nucleotide sequencing revealed that the sequence essential for suppression was surprisingly identical to *sds23*$^+$. pSDS23 indeed suppressed *nuc2-663* at 30°C (Figure 5A). The full-length *sds23*$^+$ gene was necessary for the suppression of the *nuc2* mutant (data not shown). The *sds23*$^+$ gene was the only suppressor obtained among 80 000 clones of *nuc2-663* transformants.

We examined whether plasmid pSDS23 could suppress the *cut9-665* mutations as well, as this mutant is genetically, physiologically and structurally related to *nuc2-663* (Samejima and Yanagida, 1994). *cut9* mutant cells carrying pSDS23 produced colonies at 33°C but not at 36°C, indicating that pSDS23 can suppress *cut9* but not fully (Figure 5B). These results established that the *sds23*$^+$ gene function is related to the 20S cyclosome function.

Gene disruption of sds23$^+$

To investigate whether *sds23*$^+$ was essential for viability, gene disruption was performed by one step gene replacement (Figure 6A, upper panel). Heterozygous diploids verified by Southern hybridization (lower panel) yielded four viable spores at 26 or 33°C with Ade$^+$ segregating 2$^+$:2$^-$, indicating that the *sds23*$^+$ gene was not essential for viability. Colonies were not formed at 22 or 36°C, however (Figure 6B), indicating that the gene-disrupted Δ*sds23* causes cold and temperature sensitivity. ts *sds22-181* and cs *dis2-11* strains are shown as controls.

The cell division rates of Δ*sds23* at the permissive temperatures were exceedingly slow. The generation times in rich YPD medium were 5.7 (3.3) and 4.3 (2.2) h at 26 and 33°C, respectively; the values in parenthesis are the generation times for the wild-type strain.

Gene-disrupted Δ*sds23* cells are elongated, and their shape is somewhat deformed (Δ*sds23*, Figure 7). They were stained with anti-tubulin (TUB) and anti-SPB (Sad1) antibodies. Cells were often found to contain multiple nuclei. Most of these bi- or tetra-nucleated cells appeared to be in anaphase, as the mitotic spindles were seen for some pairs of nuclei (TUB). The frequencies of cells having the short metaphase or anaphase spindle in Δ*sds23* increased from 15% at 26°C to 33% at 36°C and 5% in Δ*sds23* carrying plasmid pSDS23 (Δ*sds23*/pSDS23; shown in right panel). At 20°C, 40% of Δ*sds23* cells contained the spindle. These results showed that Δ*sds23* cells were impaired in the progression of anaphase.

The X-shaped microtubules emanating from the microtubule organizing center (MTOC) in the central position of post-anaphase cells (Hagan and Hyams, 1988) were conspicuously missing in Δ*sds23* cells. They were found normally in post-anaphase cells of Δ*sds23* carrying pSDS23 (an example of an X-shaped microtubule is indicated by the short arrow). This might be correlated with the observation that cytokinesis was greatly delayed in Δ*sds23* cells, leading to elongated cells containing multiple nuclei. Cytokinesis was normal in Δ*sds23* carrying pSDS23.

Suppression of the Δsds23 phenotype by plasmids carrying the nuc2$^+$, cut9$^+$ or sds21$^+$ gene

The gene disruption phenotypes of Δ*sds23* were partly reversed by the introduction of plasmids carrying the *nuc2*$^+$, *cut9*$^+$ or *sds21*$^+$ gene (Figure 8A). Transformants carrying plasmid pNUC2, pCUT9 or pSDS21 supported colony formation at 22°C but those carrying the vector, pDIS2 or pSDS22 did not (see below). Even at the permissive temperature (26°C), the ability of these plasmids to enhance colony formation could be clearly distinguished from that of the vector (right colored panel). Red colored colonies on the MR (phloxine B) plate contained a large number of dead cells. At 36°C, only pSDS23 was able to suppress the growth defect fully.

Transformant cells carrying different plasmids were cultured at 26°C and observed under Nomarski optics

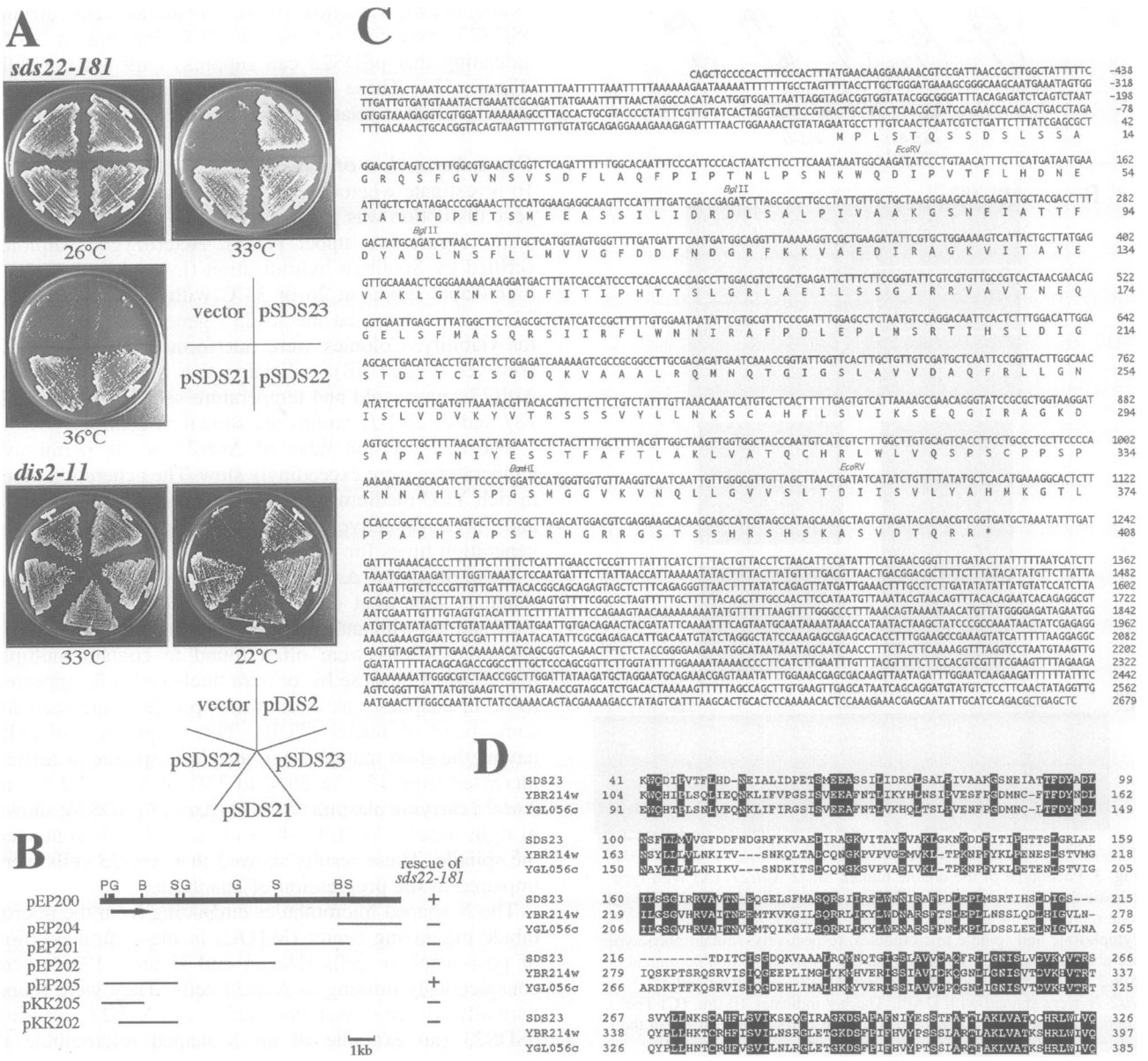

Fig. 4. Isolation of the *sds23*$^+$ gene. (**A**) Upper panel: temperature-sensitive (ts) *sds22-181* cells (Stone *et al.*, 1993) carrying vector, plasmid with the *sds21*$^+$ (pSDS21), *sds22*$^+$ (pSDS22) or *sds23*$^+$ gene (pSDS23) were plated at 26, 33 or 36°C. Cells carrying pSDS23 produced colonies at 33°C. Lower panel: cold-sensitive (cs) *dis2-11* cells carrying vector, plasmid with the *dis2*$^+$ (pDIS2), *sds23*$^+$ (pSDS23), *sds21*$^+$ (pSDS21) or *sds22*$^+$ (pSDS22) were plated at 33°C (permissive temperature) or at 22°C (restrictive temperature). pSDS23 suppressed the cs phenotype of *dis2-11*. (**B**) Subcloning of plasmid pEP200 which suppressed *sds22-181*. Plasmid pEP205 contained the minimal insert allowing complementation. The arrow indicates the coding region for *sds23*$^+$. P, *Pvu*II; G, *Bgl*II; B, *Bam*HI; S, *Sac*I. (**C**) Nucleotide sequencing and the predicted amino acid sequence of *sds23*$^+$. Some restriction sites are shown. The accession number of the sequence is D86840. (**D**) Amino acid sequence alignment among Sds23 and two open reading frames of *S.cerevisiae* (YBR214w and YGL056c). Identical amino acids are boxed.

(Figure 8B). The cell size and shape in Δ*sds23* cells carrying plasmid pNUC2 or pCUT9 became nearly normal, like those carrying pSDS23. However, transformants carrying pDIS2 and pSDS22 remained elongated and deformed as did those carrying the vector alone. Cells carrying pSDS21 also recovered from their morphological defect. These transformants appear longer than wild-type cells, but even wild-type cells carrying pSDS21 exhibited a certain cell elongation as pSDS21 was inhibitory to growth (Yamano *et al.*, 1994). Consistent with the growth suppression, the cell morphology defects of Δ*sds23* were also suppressed by elevated gene dosages of *nuc2*$^+$, *cut9*$^+$ and *sds21*$^+$, but not by *dis2*$^+$ and *sds22*$^+$. However, Δ*sds23* cells carrying plasmid pDIS2T316A with the alanine-substituted mutant *dis2* gene were able to suppress the phenotype of Δ*sds23*, as was the case for pSDS21 (data not shown).

Sds23 is a component of neither Dis2 phosphatase nor the 20S complex

Antibodies against Sds23 were prepared and employed for detecting Sds23 protein in *S.pombe* extracts by immunoblot (Materials and methods). A predicted protein band was detected at 45 kDa, and the intensity increased in cells

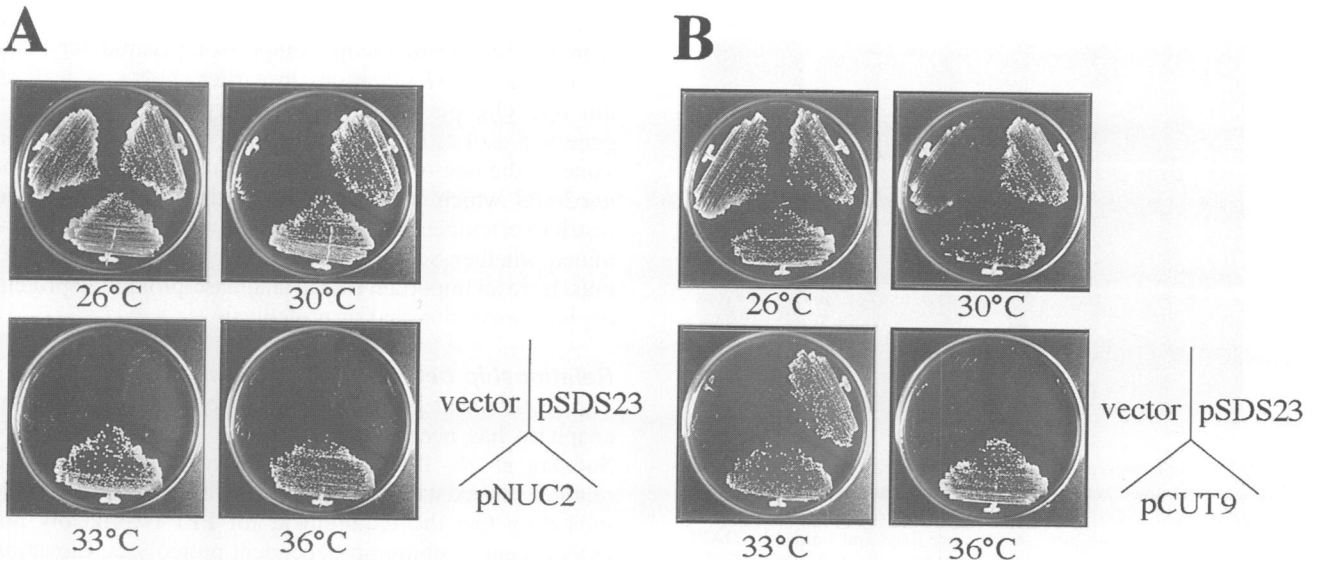

Fig. 5. pSDS23 suppresses mutants for the 20S cyclosome components. (**A**) ts *nuc2-663* mutant cells carrying the vector, pSDS23 or pNUC2 were plated at 26, 30, 33 and 36°C. pSDS23 suppressed *nuc2-663* at 30°C. (**B**) ts *cut9-665* mutant cells carrying the vector, pSDS23 or pCUT9 were plated at 26, 30, 33 and 36°C. pSDS23 suppressed *cut9-665* at 30 and 33°C.

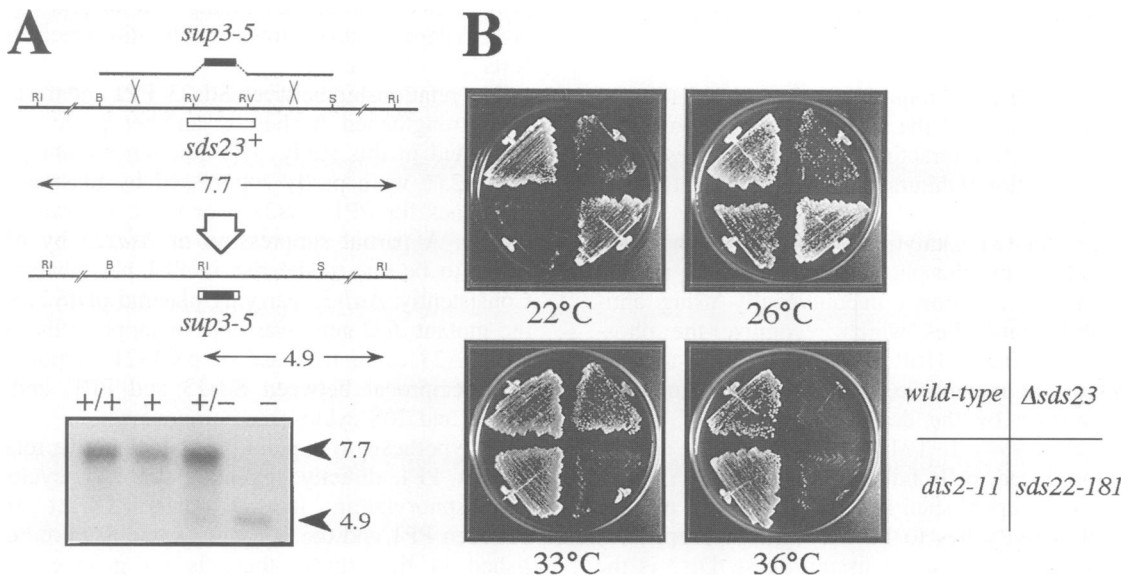

Fig. 6. Gene disruption of *sds23*$^+$. (**A**) The *sds23*$^+$ gene was disrupted by one-step gene replacement (Rothstein, 1983). The *sup3-5* gene (Hofer *et al.*, 1979) was used to replace the coding region of *sds23*$^+$ as indicated, and the resulting plasmid with the disrupted gene was used for transformation of a diploid. Heterozygous diploid (+/–) showed the expected bands (7.7 and 4.9 kb *Eco*RI bands) by genomic Southern hybridization. A haploid segregant containing the disrupted gene (–) showed only the 4.9 kb band. RI, *Eco*RI; B, *Bam*HI; RV, *Eco*RV; S, *Sac*I. (**B**) The gene-disrupted haploid segregant of *sds23*$^+$ (Δ*sds23*) produced colonies at 33°C, but not at 36°C. Tiny colonies were made at 22°C. cs *dis2-11* and ts *sds22-181* are shown as a control.

carrying a multicopy plasmid with the *sds23*$^+$ gene (Figure 9A). The band was not detected in the *sds23*-deleted cells (data not shown).

Immunoprecipitation experiments showed that Sds23 protein did not co-precipitate with Dis2 by anti-dis2 (D2F) antibodies or with Sds22 by anti-sds22 antibodies (Figure 9B), suggesting that Sds23 did not form a complex with Dis2-Sds22 phosphatase. Sds23 also did not form a complex with Sds21, as shown using antibodies which were able to immunoprecipitate Sds21 (data not shown).

A sucrose gradient centrifugation was performed to determine whether Sds23 was a component of the 20S complex. Sds23 protein sedimented at a velocity much slower than the 20S complex (Figure 9C). Cut9 and Nuc2, as expected, were components of the 20S complex (Figure 9C; H.Yamada, K.Kumada and M.Yanagida, manuscript in preparation). Sds23 thus did not exist in the 20S complex. Immunoprecipitation using anti-cut9 antibodies also confirmed that Cut9 and Sds23 do not form the complex (data not shown).

Discussion

Requirement for PP1 for exiting mitosis

We show here that the double deletion mutant of PP1 catalytic subunits in fission yeast is arrested at metaphase,

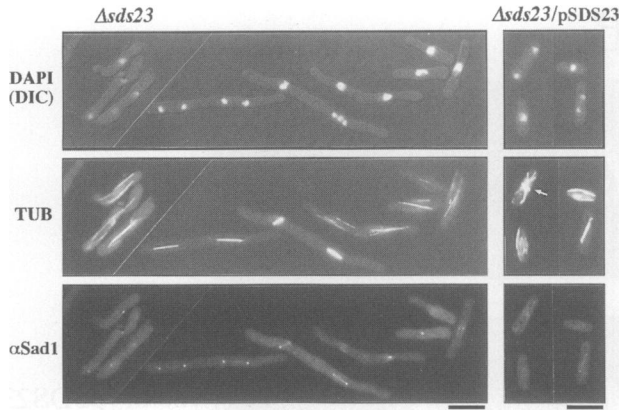

Fig. 7. Immunofluorescence micrographs of *sds23* gene-disrupted cells. *sds23* gene-disrupted cells (Δ*sds23*, left panel) and the disrupted cells carrying plasmid pSDS23 (Δ*sds23*/pSDS23) were grown at the permissive temperature (26°C). Cells were fixed and stained by DAPI (top) for nuclear DNA, anti-tubulin (TUB; Woods *et al.*, 1989) for microtubules and anti-sad1 (αSad1; Hagan and Yanagida, 1995) for SPB. Nomarski images were superimposed in the top panel. Many Δ*sds23* cells were multinucleated, contained divided nuclei and had spindles of varying lengths. Post-anaphase microtubule arrays (Hagan and Hyams, 1988) were rarely observed. An example of these arrays is indicated by the small arrow in Δ*sds23*/pSDS23 (right panel). The bars indicate 10 μm.

suggesting that PP1 is required for the initiation of anaphase. This supported the previous conclusion that a mitotic form of PP1 interacting with Sds22 is essential for anaphase initiation (Ohkura and Yanagida, 1991; Stone *et al.*, 1993).

Dis2, one of the two catalytic subunits of PP1 in fission yeast, is regulated by phosphorylation during the normal cell cycle. This was shown unequivocally using anti-phosphopeptide antibodies which recognize the phosphorylated C-terminal T316P. Dis2 phosphatase appears to be inhibited at metaphase, and reactivated upon the onset of anaphase by the dephosphorylation of T316P (Yamano *et al.*, 1994). If T316 is substituted by alanine and is overproduced, cells fail to enter mitosis (Yamano *et al.*, 1994; our unpublished results), suggesting that the level of PP1 activity has to be decreased appropriately upon the entry into mitosis in fission yeast. Dis2 is the major PP1 in fission yeast because the level of Dis2 protein in wild-type cell extracts is higher than that of Sds21 (Kinoshita *et al.*, 1990). Cdc2 kinase is likely to be responsible for this negative regulation as the T316 site is a consensus site for Cdc2 kinase. A similar cell cycle regulation of PP1 by phosphorylation has been found in other organisms (Dohadwala *et al.*, 1994).

Hyperphosphorylation of T316 also occurs when Dis2 is overproduced. This is perhaps the reason why pDIS2 does not suppress the deletion of *sds23*$^+$, since Dis2 activity is down-regulated by phosphorylation. pSDS21 can suppress this deletion, however, as Sds21 is not phosphorylated *in vivo* and the PP1 activity increases with the increase in *sds21*$^+$ gene dosage (Yamano *et al.*, 1994). This case is similar to that of Δ*sds22*, which can be suppressed by pSDS21 but not by pDIS2 (Ohkura and Yanagida, 1991).

The importance of PP1 in the initiation of anaphase was thus established, but its actual role in anaphase is poorly understood. We therefore wanted to identify the major substrate for PP1. As one attempt to do so, we searched for the multicopy suppressor plasmid for *dis2-11* and *sds22-181* mutations in earlier studies as well as this one. One plasmid, pSDS23, carrying a novel *sds23*$^+$ gene was isolated and, surprisingly, turned out to be the same as the one obtained from an independent screen for *nuc2-663* which was also blocked in metaphase at the restrictive temperature. Although it remains to be determined whether Sds23 protein is the substrate for PP1, it must have an important role in anaphase-promoting protein dephosphorylation and/or proteolysis.

Relationship between PP1 and the cyclosome

The requirement for the 20S cyclosome for initiation of anaphase has been well established (King *et al.*, 1995; Sudakin *et al.*, 1995). We show here that a functional relationship exists between the 20S cyclosome and PP1, indicating that the requirement for PP1 is probably not independent of ubiquitin-dependent proteolysis. Elevation of the *sds23*$^+$ gene dosage lessens the requirement for PP1 and the 20S cyclosome for the onset of anaphase, suggesting that Sds23 directly or indirectly interacts with PP1 and/or the 20S cyclosome. The *sds23*$^+$ gene hence provides a clue to understanding how anaphase-promoting protein dephosphorylation and ubiquitin-dependent proteolysis are interrelated.

The relationship between Sds23, PP1 and the cyclosome was strengthened further by another piece of evidence presented in this study. The deletion mutant phenotypes of *sds23*$^+$ were partly suppressed by plasmids carrying the genes for PP1 (*sds21*$^+$) or the cyclosome (*nuc2*$^+$ or *cut9*$^+$). A partial suppression of Δ*sds23* by pSDS21 is likely to be due to the rise in PP1 phosphatase activity. Consistently, Δ*sds23* carrying plasmid pDIS2T316A with the mutant *dis2* gene was able to suppress the phenotype of Δ*sds23* as in the case of pSDS21. Suppression was thus reciprocal between Sds23 and PP1, and between Sds23 and 20S cyclosome components.

A hypothesis to explain part of the above relationships is that PP1 directly regulates the 20S cyclosome by dephosphorylating its subunit(s). Direct interaction between PP1 and the 20S cyclosome has not been established in this study: there is no genetic suppression between PP1 and the cyclosome/APC subunit genes (our unpublished results). However, *dis2-11* and *cut9-665* are synthetically lethal (H.Yamada, K.Kumada and M.Yanagida, manuscript in preparation), suggesting that PP1 and Cut9 might share an essential function. Cut9 is a phosphoprotein, the phosphorylation level of which changes during the cell cycle: rapid dephosphorylation occurs in anaphase (H.Yamada, K.Kumada and M.Yanagida, manuscript in preparation). Whether PP1 dephosphorylates Cut9, and whether Cut9 dephosphorylation regulates the activity of the 20S complex is not yet known, however. Similarly, in *Xenopus*, the components of the cyclosome are also phosphorylated (King *et al.*, 1995).

Possible roles of Sds23 in initiating anaphase

The actual molecular function of Sds23 is elusive. The amino acid sequence does not contain any known motif found in phosphatases or proteases, but shows a considerable similarity to two hypothetical proteins (YBR214w and YGL056c) in *S.cerevisiae* identified by genome analysis.

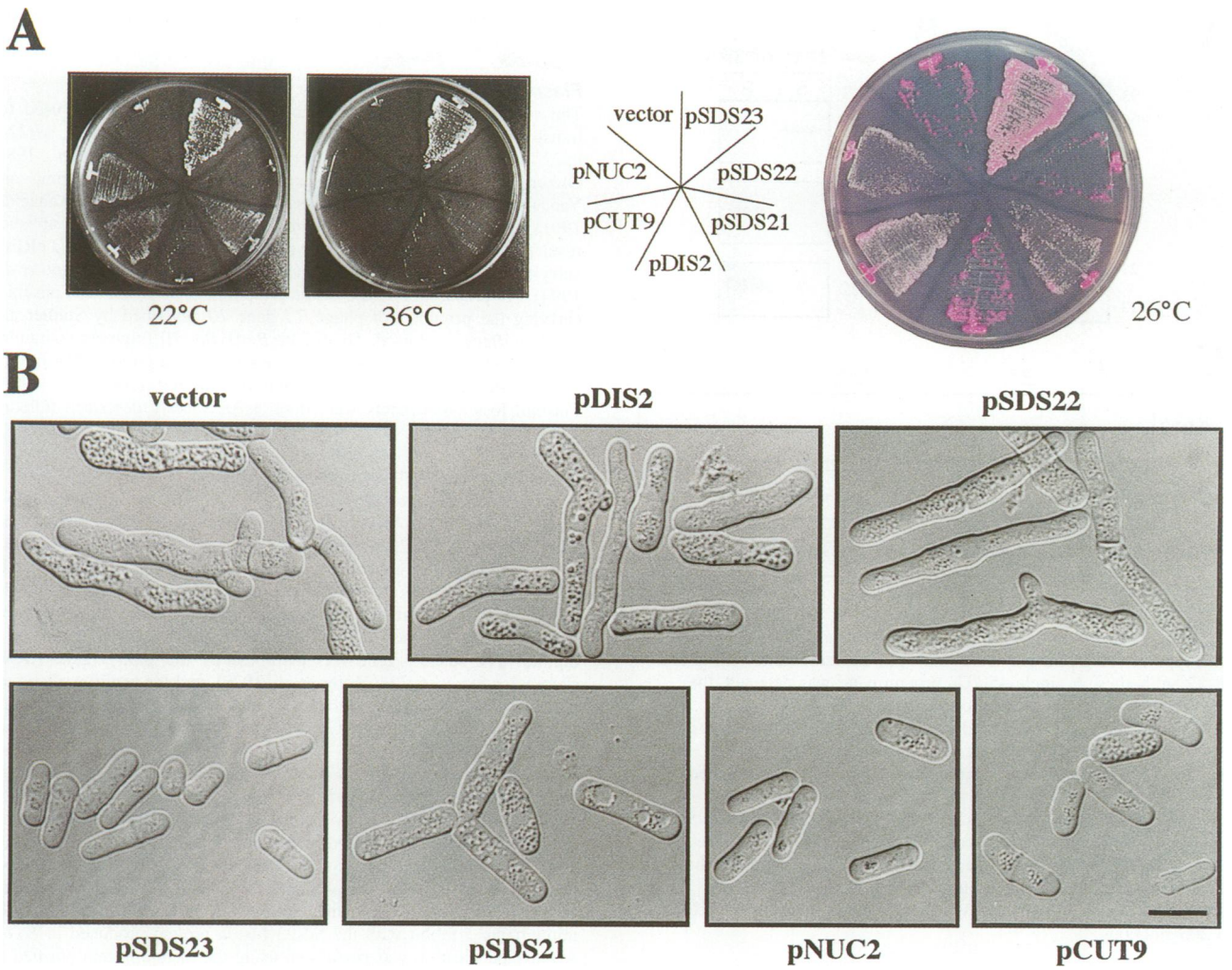

Fig. 8. Suppression of Δ*sds23* by pNUC2, pCUT9 and pSDS21. (**A**) Δ*sds23* cells carrying the vector, pSDS23, pSDS22, pSDS21, pDIS2, pCUT9 or pNUC2 were plated at 22, 26 (color, right panel) and 36°C. pSDS23 suppressed Δ*sds23* at all temperatures. pSDS21, pNUC2 and pCUT9 partly suppressed Δ*sds23* at 22°C. At 26°C, Δ*sds23* carrying pSDS23, pSDS21, pNUC2 or pCUT9 produced white colonies on the MR plate, while others containing numerous dead cells produced red colonies on the same plate. (**B**) Nomarski light micrographs of Δ*sds23* cells carrying the vector, pSDS23, pDIS2, pSDS21, pSDS22, pNUC2 or pCUT9. The bar indicates 10 μm.

Comparison of the fission yeast and budding yeast Sds23 sequences suggests that the central domains are conserved, while the terminal regions are not homologous but are both hydrophilic and basic in the C-terminal region.

Immunoprecipitation experiments demonstrated that Sds23 was bound neither to Dis2 (the present study) nor to Sds21 and Sds22 (our unpublished result). Sucrose gradient centrifugation also showed that Sds23 was not a component of the 20S complex. Thus, it is unlikely that Sds23 is a regulatory subunit for PP1 holoenzymes or a subunit of the cyclosome. Preliminary immunofluorescence microscopy using anti-sds23 suggested that Sds23 was diffused throughout the cell (our unpublished result). Sds23 seems to function as a form distinct from PP1 and the cyclosome.

Sds23 is not absolutely required for cell viability: the deletion mutant was cold-sensitive and also temperature-sensitive, but grew only very poorly at the permissive temperature. It remains to be determined whether there might be another gene similar to Sds23 as in budding yeast. Gene disruption of *sds23*$^{+}$ showed pleiotropic defects. Dividing cells were often elongated and somewhat abnormal in shape at both permissive and restrictive temperatures, and contained multiple nuclei. The cytoskeletal architecture was aberrant, and cell separation was greatly delayed. These various abnormalities were also observed at both the permissive and restrictive temperatures. Anti-tubulin staining revealed that the frequencies of cells containing the short or elongating spindle with divided nuclei were very high, suggesting that anaphase was greatly retarded. One of the Sds23 functions clearly is to facilitate the progression in anaphase, but Sds23 protein appears to be involved in at least two other major cellular events as well, i.e. cytokinesis and cell shape control.

Sds23 possibly may regulate the 20S cyclosome in an unknown fashion, controlling its substrate specificity or cell cycle-dependent activity or structure. Alternatively, it might mediate target proteins for proteolysis to interact with the 20S cyclosome. Cyclins have the sequences required for destruction, called the destruction box (Glotzer, 1995); Sds23 might interact with such destruction box sequences. Other possibilities are that Sds23 may control mitotic PP1 and vice versa or that Sds23 is a

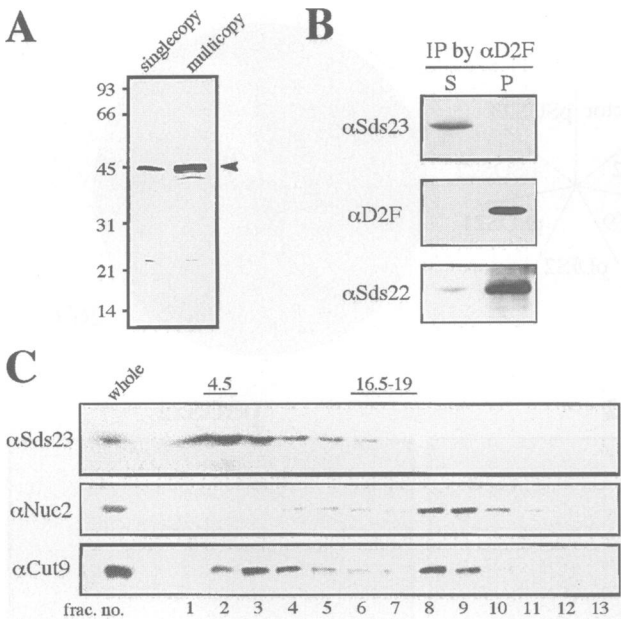

Fig. 9. Identification of Sds23 protein. (**A**) Extracts of wild-type and wild-type carrying pSDS23 were made and immunoblotted using anti-sds23 antibodies. A single 45 kDa protein band was detected, the intensity of which increased in cells carrying pSDS23. (**B**) Immunoprecipitation of wild-type extracts was performed using anti-dis2 antibodies (D2F; Stone *et al.*, 1993). Resulting immunoprecipitates (P) and supernatants (S) were immunoblotted by anti-sds23, anti-dis2 and anti-sds22 antibodies. Sds23 did not co-precipitate with Dis2, while Sds22 did. (**C**) Wild-type cell extracts were run in a sucrose gradient centrifugation as described in Funabiki *et al.* (1996), and fractions collected were analyzed by immunoblotting using anti-sds23, anti-nuc2 and anti-cut9 antibodies. Sds23 was detected mostly in supernatant, whereas Nuc2 and Cut9 were detected in 20S fractions.

target of the 20S complex. It has not been determined whether Sds23 is ubiquitinated or phosphorylated.

An unexpected finding was that plasmids carrying *nuc2*$^+$ and *cut9*$^+$ completely suppressed cell shape abnormality and the cytokinesis defect in the *sds23* deletion mutant. Nuc2 and Cut9 proteins do not appear to function solely at the onset of anaphase. These 'extra' functions might be due to the excess of the 20S particle or free Nuc2 and Cut9 proteins. Massive overproduction of Nuc2 under the *nmt1* promoter leads to the complete blockage of cytokinesis (Kumada *et al.*, 1995). The 20S cyclosome may mediate a number of proteins other than cyclins for proteolysis. An example of such a protein is Cut2, which is essential for sister chromatid separation (Funabiki *et al.*, 1996). Proteins required for maintaining normal cell shape or promoting cytokinesis might also be the target of proteolysis dependent upon the 20S cyclosome. Diverse functions for the 20S cyclosome are now further supported by a recent publication which demonstrates the role of the 20S cyclosome in regulating the property of DNA replication once per cell cycle (Heichman and Roberts, 1996).

Materials and methods

Strains and media

Schizosaccharomyces pombe strains used were described previously (Hirano *et al.*, 1986, 1988; Ohkura *et al.*, 1988, 1989; Stone *et al.*, 1993; Yamano *et al.*, 1994). The complete YPD medium (1% yeast extract, 2% bactopeptone and 2% glucose) and the minimal EMM2 (Mitchison, 1970) were used.

Plasmids

The shuttle vector pDB248′ (Beach and Nurse, 1981) was used for transformation of *S.pombe*. Plasmids carrying *dis2*$^+$, *sds21*$^+$, *sds22*$^+$, *nuc2*$^+$ and *cut9*$^+$ genes were described previously (Hirano *et al.*, 1988; Ohkura *et al.*, 1989; Ohkura and Yanagida, 1991; Samejima and Yanagida, 1994). The previously assigned *sds23*$^+$ (Ohkura and Yanagida, 1991) turned out to be a partly deleted *sds22* gene (our unpublished result). For overproduction of wild-type and mutant Dis2, plasmid pREP1 carrying the *nmt1* promoter (Maundrell, 1990) was used (Yamano *et al.*, 1994). For preparation of the fusion protein in *Escherichia coli*, plasmids carrying the promoter of phage T7 gene *10* described by Studier and Moffat (1986) were used. The 0.7 kb *Bgl*II–*Bam*HI fragment containing a portion of *sds23*$^+$ was used to express the fusion protein. The lithium method was used for transformation of *S.pombe* (Ito *et al.*, 1983). Plasmid loss experiments were done as previously described (Ohkura and Yanagida, 1991).

Isolation of sds23$^+$ and gene disruption

Transformants of *S.pombe h*$^+$ *sds22-181 leu1 his2* by a genomic DNA library were replica-plated at 33°C, and plasmids were recovered from those grown at 33°C and examined by Southern hybridization using *sds21*$^+$ and *sds22*$^+$ as the probes. One plasmid carried neither the *sds21*$^+$ nor the *sds22*$^+$ gene. The plasmid carrying the minimal complementable DNA insert was designated pSDS23. Gene disruption was performed by one-step gene replacement (Rothstein, 1983) using a diploid. The *sds23*$^+$ gene was disrupted by the *sup3-5* gene (tRNA suppressor for *ade6-704*; Hofer *et al.*, 1979), and the resulting disrupted *sds23* gene was integrated onto the chromosome by homologous recombination. The resulting heterozygous diploids were sporulated and tetrads were dissected.

Immunochemical methods

Anti-T316P antiserum was obtained by injecting the phosphopeptide NWHMTP^{P}PRKN conjugated with hemocyanin. Anti-D2F antibodies were described previously (Stone *et al.*, 1993). Anti-cdc13 was made by Dr I.Hagan. TAT1 and PSTAIR were the gifts of Drs K.Gull, M.Yamashita and Y.Nagahama. Anti-sds23 antibodies were made by immunizing a rabbit with the Sds23 fusion protein produced in *E.coli*. Affinity purification was performed using the fusion protein purified by fractionation and SDS–PAGE followed by electroelution. Immunoblotting was done according to the standard procedure (Towbin *et al.*, 1979). The procedures for immunoprecipitation adapted for *S.pombe* extracts were described previously (Stone *et al.*, 1993; Yamano *et al.*, 1994). Briefly, antibodies were adsorbed by incubation for 2 h with previously swollen protein A–Sepharose (Pharmacia) and washed several times in the buffer used for the swelling. Then, the antibodies complexed with Sepharose were incubated with the *S.pombe* extracts (described below) for 3–6 h followed by washing.

Preparation of S.pombe extracts

The *S.pombe* cells were broken by vigorous agitation with glass beads. The supernatants after centrifugation at 2000 *g* for 5 min or 14 000 *g* for 20 min were used as the extracts. Two kinds of buffers were employed: TEG buffer for regular use [50 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl flouride (PMSF) and 10% glycerol]; and HB containing inhibitors of phosphatases and proteases to maintain the phosphorylated state [25 mM Tris–HCl (pH 7.5) containing 15 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 0.5 mM Na_3VO_4, 1 mM dithiothreitol, 0.1% NP-40, 1 mM PMSF, 20 mg/ml soybean trypsin inhibitor, 20 mg/ml TPCK, 50 mg/ml leupeptin and 2 mg/ml aprotinin]. An appropriate concentration of NaCl was added when required for extraction. The H1 kinase activity in *S.pombe* extracts was measured by the procedure described previously (MacNeil and Fantes, 1993).

Preparation of the spores deleting both dis2$^+$ and sds21$^+$

Two double gene disruptants (DD1: *h*$^-$ *leu1 ura4 dis2::ura4*$^+$ *sds21::ura4*$^+$ and DD2: *h*$^+$ *leu1 his2 ura4 dis2::ura4*$^+$ *sds21::ura4*$^+$) were lethal but their transformant strains DD1 and DD2 carrying plasmid pDIS2 with *dis2*$^+$ or plasmid pSDS21 with *sds21*$^+$, respectively, were viable (Ohkura *et al.*, 1989). The vector plasmid contained the *S.cerevisiae LEU2* as the marker. These transformant strains were crossed and sporulated, and the resulting spores were germinated on a non-selective medium (EMM2 supplemented with Leu and His). Plasmids

were lost in certain spores (~30%) during sporulation, judging from the number of Leu$^+$ colonies estimated by plating the spores on a minimal plate. Spores were germinated on the non-selective medium and DAPI stained after 20 h at 30°C. Among 125 spores examined, 12 did not germinate, while 85 germinated and produced microcolonies consisting of 8–16 normally growing cells. These represent cells carrying the plasmid. The remaining 26 showed cells arrested in mitosis with condensed chromosomes.

Light microscopy

Immunolocalization was performed using anti-tubulin and anti-sad1 antibodies (Woods *et al.*, 1989; Hagan and Yanagida, 1995) based on the procedures previously described (Hagan and Hyams, 1988). The fixation method using glutaraldehyde was employed.

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References

Axton,J.M., Dombradi,V., Cohen,P.T.W. and Glover,D.M. (1990) One of the protein phosphatase 1 isozymes in *Drosophila* is essential for mitosis. *Cell*, **63**, 33–46.

Beach,D. and Nurse,P. (1981) High-frequency transformation of the fission yeast *Schizosaccharomyces pombe*. *Nature*, **290**, 140–142.

Dohadwala,M., da Cruz e Silva,E.F., Hall,F.L., Williams,R.T., Carbonard-Hall,D.A., Nairn,A.C., Greengard,P. and Berndt,N. (1994) Phosphorylation and inactivation of protein phosphatase I by cyclin-dependent kinases. *Proc. Natl Acad. Sci. USA*, **91**, 6408–6412.

Doonan,J.H. and Morris,N.R. (1989) The *bimG* gene of *Aspergillus*, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1. *Cell*, **57**, 987–996.

Funabiki,H., Yamano,H., Kumada,K., Nagao,K., Hunt,T. and Yanagida,M. (1996) Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature*, **381**, 438–441.

Ghislain,M., Udvardy,A. and Mann,C. (1993) *S.cerevisiae* 26S protease mutants arrest cell division in G2/metaphase. *Nature*, **366**, 358–361.

Glotzer,M. (1995) The only way out of mitosis. *Curr. Biol.*, **5**, 970–972.

Glotzer,M., Murray,A.W. and Kirschner,M.W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature*, **349**, 132–138.

Goebl,M. and Yanagida,M. (1991) The TPR snap helix: a novel protein repeat motif from mitosis to transcription. *Trends Biochem. Sci.*, **16**, 173–177.

Gordon,C., McGurk,G., Dillon,P., Rosen,C. and Hastie,N.D. (1993) Defective mitosis due to a mutation in the gene for a fission yeast 26S protease subunit. *Nature*, **366**, 355–357.

Hagan,I. 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.

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.

Heichman,K.A. and Roberts,J.M. (1996) The yeast CDC16 and CDC27 genes restrict DNA replication to once per cell cycle. *Cell*, **85**, 39–48.

Hershko,A., Ganoth,D., Pehrson,D., Palazzo,R. and Cohen,L.H. (1991) Methylated ubiquitin inhibits cyclin degradation in clam embryo extracts. *J. Biol. Chem.*, **266**, 16376–16379.

Hershko,A., Ganoth,D., Sudakin,V., Dahan,A., Cohen,L.H., Luca,F.C., Ruderman,J.V. and Eytan,E. (1994) Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase cdc2. *J. Biol. Chem.*, **269**, 4940–4946.

Hirano,T., Funahashi,S., Uemura,T. and Yanagida,M. (1986) Isolation and characterization of *Schizosaccharomyces pombe cut* mutants that block nuclear division but not cytokinesis. *EMBO J.*, **5**, 2973–2979.

Hirano,T., Hiraoka,Y. and Yanagida,M. (1988) A temperature sensitive mutation of the *S.pombe* gene *nuc2*$^+$ that encodes a nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase. *J. Cell Biol.*, **106**, 1171–1183.

Hirano,T., Kinoshita,N., Morikawa,K. and Yanagida,M. (1990) Snap helix with knob and hole and joint; essential repeats in *S.pombe* nuclear protein *nuc2*$^+$. *Cell*, **60**, 319–328.

Hisamoto,N., Frederick,D.L., Sugimoto,K., Tatchell,K. and Matsumoto,K. (1995) The *EGP1* gene may be a positive regulator of protein phosphatase type 1 in the growth control of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **15**, 3767–3776.

Hofer,F., Hollenstein,H., Janner,F., Minet,M., Thuriaux,P. and Leupold,U. (1979) The genetic fine structure of nonsense suppressor in *Schizosaccharomyces pombe*. I. sup3 and sup9. *Curr. Genet.*, **1**, 45–61.

Holloway,S., Glotzer,M., King,R.W. and Murray,A.W. (1993) Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell*, **73**, 1393–1402.

Irniger,S., Piatti,S. and Nasmyth,K. (1995) Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell*, **81**, 269–278.

Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163–168.

King,R.W., Peters,J.-M., Tugendreich,S., Rolfe,M., Hieter,P. and Kirschner,M.W. (1995) A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell*, **81**, 279–288.

Kinoshita,N., Ohkura,H. and Yanagida,M. (1990) Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle. *Cell*, **63**, 405–415.

Kinoshita,N. *et al.* (1991) Sister-chromatid separation and protein dephosphorylation in mitosis. *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 621–628.

Kumada,K., Su,S., Yanagida,M. and Toda,T. (1995). Fission yeast TPR-family protein nuc2 is required for G1-arrest upon nitrogen starvation and is an inhibitor of septum formation. *J. Cell Sci.*, **108**, 895–905.

Lamb,J.R., Michaud,W.A., Sikorski,R.S. and Hieter,P.A. (1994) Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *EMBO J.*, **13**, 4321–4328.

MacKelvie,S.H., Andrews,P.D. and Stark,M.J.R. (1995) The *Saccharomyces cerevisiae* gene *SDS22* encodes a potential regulator of the mitotic function of yeast type 1 protein phosphatase. *Mol. Cell. Biol.*, **15**, 3777–3785.

MacNeil,S.A. and Fantes,P.A. (1993) Methods for analysis of the fission yeast cell cycle. In Fantes,P. and Brooks,R. (eds), *The Cell Cycle: A Practical Approach*. IRL Press at Oxford University Press, Oxford, UK, pp. 93–125.

Maundrell,K. (1990) *nmt1* of fission yeast. *J. Biol. Chem.*, **265**, 10857–10864.

Mitchison,J.M. (1970) Physiological and cytological methods for *Schizosaccharomyces pombe*. *Methods Cell Physiol.*, **4**, 131–165.

Mizukami,T. *et al.* (1993) A 13 kb resolution cosmid map of the 14 Mb fission yeast genome by nonrandom sequence-tagged site mapping. *Cell*, **73**, 121–132.

Nabeshima,K., Kurooka,H., Takeuchi,M., Kinoshita,K., Nakaseko,Y. and Yanagida,M. (1995) p93^{dis1}, which is required for sister chromatid separation, is a novel microtubule and spindle pole body-associating protein phosphorylated at the Cdc2 target sites. *Genes Dev.*, **9**, 1572–1585.

Ohkura,H. and Yanagida,M. (1991) *S.pombe* gene *sds22*$^+$ essential for a midmitotic transition encodes a leucine-rich repeat protein that positively modulates protein phosphatase-1. *Cell*, **64**, 149–157.

Ohkura,H., Adachi,Y., Kinoshita,N., Niwa,O., Toda,T. and Yanagida,M. (1988) Cold-sensitive and caffeine supersensitive mutants of the *Schizosaccharomyces pombe* dis genes implicated in sister chromatid separation during mitosis. *EMBO J.*, **7**, 1465–1473.

Ohkura,H., Kinoshita,N., Miyatani,S., Toda,T. and Yanagida,M. (1989) The fission yeast *dis2*$^+$ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. *Cell*, **57**, 997–1007.

Renouf,S., Beullens,M., Wera,S., Van Eynde,A., Sikela,J., Stalmans,W. and Bollen,M. (1995) Molecular cloning of a human polypeptide related to yeast sds22, a regulator of protein phosphatase-1. *FEBS Lett.*, **375**, 75–78.

Rothstein,R.J. (1983) One-step gene disruption in yeast. *Methods Enzymol.*, **101**, 202–211.

Samejima,I. and Yanagida,M. (1994) Bypassing anaphase by fission yeast *cut9* mutation: requirement of *cut9*$^+$ to initiate anaphase. *J. Cell Biol.*, **127**, 1655–1670.

Sikorski,R.S., Boguski,M.S., Goebl,M. and Hieter,P. (1990) A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. *Cell*, **60**, 307–317.

Stone,E.M., Yamano,H., Kinoshita,N. and Yanagida,M. (1993) Mitotic regulation of protein phosphatases by the fission yeast sds22 protein. *Curr. Biol.* **3**, 13–26.

Studier,F.W. and Moffat,B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.*, **189**, 113–130.

Sudakin,V., Ganoth,D., Dahan,A., Heller,H., Hershko,J., Luca,F., Ruderman,J.V. and Hershko,A. (1995) The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Mol. Biol. Cell*, **6**, 185–198.

Surana,U., Amon,A., Dowzer,C., McGrew,J., Byers,B. and Nasmyth,K. (1993) Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.*, **12**, 1969–1978.

Towbin,H., Staehelin,T. and Gordon,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedure and some applications. *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.

Tugendreich,S., Tomkiel,J., Earnshaw,W. and Hieter,P. (1995) The CDC27HS protein co-localizes with the CDC16HS protein to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. *Cell*, **81**, 261–268.

Wilson,R. *et al.* (1994) 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C.elegans*. *Nature* **368**, 32–38.

Woods,A., Sherwin,T., Sasse,R., McRae,T.H., Baines,A.J. and Gull,K. (1989) Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell Sci.*, **93**, 491–500.

Yamano,H., Ishii,K. and Yanagida,M. (1994) Phosphorylation of dis2 protein phosphatase at the C-terminal cdc2 consensus and its potential role in cell cycle regulation. *EMBO J.*, **13**, 5310–5318.

Yanagida,M. (1989) Gene products required for chromosome separation. In Brooks,P., Fantes,P., Hunt,T. and Wheatley,D. (eds), *The Cell Cycle. Journal of Cell Science Supplement*. The Company of Biologists, Cambridge, UK, pp. 213–229.

Yanagida,M. (1995) Frontier questions about sister chromatin separation in anaphase. *BioEssays*, **17**, 519–526.

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