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High dosage expression of a zinc finger protein, Grt1, suppresses a mutant of fission yeast *slp1*$^{+}$, a homolog of CDC20/p55CDC/Fizzy

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SUMMARY

Selective proteolysis at and after the onset of anaphase is a key cell cycle event required for sister chromatid separation as well as for exit from mitosis. It requires ubiquitination of substrates by Anaphase Promoting Complex(APC)/ Cyclosome. Slp1, a WD-repeat protein, is a putative activator for APC in fission yeast. With another WD-repeat protein, Ste9/Srw1, it is thought to promote the proteolysis in a substrate-specific manner. We report here characterization of a temperature-sensitive (ts) *slp1* mutant and its high-dosage suppressor, *grt1*$^{+}$. In cells arrested in metaphase, wild-type Slp1 was preferentially found in a complex with hyperphosphorylated Cut9 (subunit of APC), whereas the ts Slp1 protein, lacking the last 113 amino acids, failed to interact with Cut9. The temperature sensitivity was suppressed by high dosage expression of a zinc finger protein, Grt1. The ts *slp1* mutant was unable to maintain the normal level of Grt1 protein. The reduction in the Grt1 level may be a primary defect since high dosage expression of *grt1*$^{+}$ rescues the *slp1* mutant. The grt1-suppression had an additive effect to Δ*ste9* and *wee1-50*, both of which partially suppress the ts *slp1* mutant. Therefore, *grt1*$^{+}$ would define an independent pathway that facilitates the function of Slp1.

Key words: Anaphase Promoting Complex (APC), Slp1/CDC20, Zinc finger, Mitosis, Fission yeast

INTRODUCTION

Genetic and biochemical evidence has indicated that cell cycle progression through mitosis requires ubiquitin-dependent proteolysis. A defect in the 26S proteasome, which is responsible for degradation of ubiquitinated proteins, causes a metaphase arrest in yeast mutants (Ghisiain et al., 1993; Gordon et al., 1993; Gordon et al., 1996). Inhibition of ubiquitin-dependent proteolysis in egg extracts delays cyclin A and B degradation and exit from mitosis (Hershko et al., 1991). Mitotic cyclin, a subunit of the CDK kinase, is one of the substrates for the ubiquitin-dependent proteolysis during exit from mitosis. A short amino acid motif of the mitotic cyclins, denoted the destruction box, has been identified as necessary for the proteolysis. A modified form of mitotic cyclin, which lacks the destruction box and thus is resistant to the proteolysis, causes a delay in exit from mitosis (Murray et al., 1989; Glotzer et al., 1991), but not sister chromatid separation, suggesting the presence of additional proteins to be degraded prior to the onset of sister chromatid separation (Holloway et al., 1993).

Ubiquitination proceeds in a stepwise fashion and requires multiple enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3) (for reviews, see Hershko, 1997; Hochstrasser, 1995, Hochstrasser, 1996). A large protein complex, Anaphase Promoting Complex (APC)/Cyclosome, serves as an E3 enzyme for ubiquitination of mitotic cyclins (King et al., 1995; Sudakin et al., 1995; for reviews, see Townsley and Ruderman, 1998; Peters, 1998; Morgan, 1999; Zachariae and Nasmyth, 1999). The complex contains at least 12 components in budding yeast (Zachariae et al., 1996). Mutants defective in APC are unable to degrade mitotic cyclins (Irniger et al., 1995; Zachariae and Nasmyth, 1996). They also fail to initiate sister chromatid separation, implying that APC is responsible for ubiquitinating mitotic cyclins as well as proteins to be degraded for sister chromatid separation (Irniger et al., 1995). In fission yeast seven subunits of APC/Cyclosome have so far been identified (reviewed in Yanagida, 1998; Yanagida et al., 1999). Temperature-sensitive (ts) mutants of the APC subunits, namely *cut4*, *cut9* and *nuc2*, cause a delay in the onset of anaphase (Yamashita et al., 1996; Samejima and Yanagida, 1994; Hirano et al., 1988). It has also been shown that the APC is disassembled in these mutants upon shift to the restrictive temperature (Yamashita et al., 1996; Yamada et al., 1997).

Fission yeast Cut2 and its budding yeast homolog, Pds1, are other proteins that are degraded in an ubiquitin-dependent manner at the transition from metaphase to anaphase (cut2: Funabiki et al., 1996a, Funabiki et al., 1996b, Funabiki et al., 1997; Pds1: Cohen-Fix et al., 1996; Yamamoto et al., 1996). Upon removal of the destruction box, the Cut2/Pds1 protein becomes stable. Proteolysis of Cut2/Pds1 is required for sister chromatid separation because expression of mutant Cut2/Pds1 lacking the destruction box blocks sister chromatid separation

(Funabiki et al., 1997). In addition to mitotic cyclins and Cut2/Pds1, budding yeast Ase1, Cdc20 and Cdc5 proteins are degraded in and after mitosis (Juang et al., 1997; Shirayama et al., 1998; Prinz et al., 1998). It has been demonstrated in budding yeast that the temporal order of proteolysis of these proteins is determined by a pair of homologous proteins, Cdc20 and Cdh1/Hct1, both of which contain seven repeats of WD40 (Sethi et al., 1991; Schwab et al., 1997; Visintin et al., 1997). A mutation in CDC20 stabilizes Pds1, but not mitotic cyclin or Ase1. In a strain lacking Cdh1/Hct1, cyclin and Ase1 remain stable, but Pds1 can be degraded as normal. Overexpression of either gene causes degradation of its target protein. Thus, Cdc20 and Cdh1/Hct1 are thought to activate APC in a substrate-specific manner.

Fission yeast Slp1 protein is structurally homologous to Cdc20 (Matsumoto, 1997). As with a defect in CDC20 protein of budding yeast (Sethi et al., 1991), a ts mutation of Slp1, *slp1-362*, causes metaphase arrest at the nonpermissive temperature. Furthermore, both Slp1 and Cdc20 bind to Mad2 (He et al., 1997), a component of the spindle checkpoint (reviewed in Hardwick, 1998; Amon 1999), and serve as a target of the checkpoint (Kim et al., 1998; Hwang et al., 1998). A fission yeast protein, Srw1/Ste9, is thought to be a homolog of budding yeast Cdh1/Hct1 (Yamaguchi et al., 1997; Kitamura et al., 1998). In support of this, overexpression of *srw1*$^+$*/ste9*$^+$ induces proteolysis of mitotic cyclin (Kitamura et al., 1998). In this study, the ts Slp1-362 protein was characterized biochemically, and a novel zinc finger protein was isolated whose overexpression can suppress the growth defect of the *slp1* mutant.

MATERIALS AND METHODS

Media, culture conditions and strains

The *S. pombe* strains used in this study are listed in Table 1. Handling of *S. pombe* strains was as described by Gutz et al. (1974) and Moreno et al. (1991). For the block and release experiment of *cdc25* mutant, the procedures described by Alfa et al. (1993) were followed. Temperature-sensitive strains were cultured at the restrictive temperature (36°C) for 4 hours except for *cdc10* (3.5 hours), while *nda3* mutants were incubated at 20°C for 6 hours. YEA was used as a rich medium and PMA as a minimal medium. For the experiment shown in Fig. 7A, each cell cycle mutant was transformed by *pgrt1-334HAC*, which expresses C-terminal HA-tagged Grt1 under its authentic promoter, and the cells were arrested by shifting to a restrictive temperature (36°C or 20°C). For *nda3* and *cut4* mutants, which show medium dependency on the phenotype (Hiraoka et al., 1984; Yamashita et al., 1996), YPD was used as a rich medium containing 2% glucose, 1% yeast extract and 2% polypeptone (Waco Co., Japan). After growing the cells in the selected conditions, the medium was replaced by YPD immediately before the temperature shift.

Analysis of the mutation site of *slp1-362*

PCR was performed with the template prepared from the wild-type *972* strain or a *slp1-362* mutant. The PCR products containing the *slp1*$^+$ coding region were sequenced directly. Sequences of the oligo nucleotides used for amplification are as follows: OL-1: 5′-CCCATATGGCTGATAGTGGTTCGG-3′; OL-2: 5′-GGGCGGC-CGCCGGGAATCGAAGATCTGGC-3′; OL-4: 5′-GGGCGGCCGC-CACGGATTGTTATGCTGC-3′; OL-5: 5′-CCCATATGGTGCGTAT-CGCTAATC-3′. OL-2 and OL-4 contain a *Not*I linker sequence, and OL-1 and OL-5 contain an *Nde*I linker sequence.

Gene cloning

slp1-362 was transformed with a fission yeast genomic DNA library (a gift from Dr Mitsuhiro Yanagida, Kyoto University, Japan). The resulting transformants were first incubated at 26°C for 1 day and then at 32°C or 36°C for 3-5 days. From more than 100,000 colonies screened, approximately 150 clones were identified as primary candidates for suppressors. Among them, ten clones showed cosegregation of the plasmid marker (LEU2) and complementation at 32°C. By restriction analysis of the plasmids that were recovered from the ten clones, they were classified into six different groups. One of them, containing *grt1*$^+$, is reported here and the other groups elsewhere. The genomic DNA fragment containing *grt1*$^+$ was subcloned into pSP1 vector and designated *pSP1-334*. Analysis of the transcript by 5′-RACE was performed using the reagents and protocol provided by the manufacturer (GibcoBRL), and revealed two introns near the 5′ terminus of the gene. Total RNAs from fission yeast were prepared by using the SV total RNA isolation system (Promega).

Gene disruption and other plasmid construction

The procedure described by Grimm et al. (1988) was followed. A 1.8 kb *Bam*HI fragment containing the *ura4*$^+$ gene was inserted into the *Bgl*II site (shown in Fig. 5B) within the *grt1*$^+$ gene, which was subcloned into pUC119. A 3.5 kb *Sac*I-*Hin*dIII fragment was released by the restriction digest and used for transformation of a diploid strain, AE357. Stable Ura+ colonies were selected and the disruption was confirmed by Southern blot analysis (not shown). For Myc8his6 tagging to the C terminus of Grt1, a plasmid, *pYC11-Mad2C-myc8his6* (a gift from Dr Mitsuhiro Yanagida), was employed. On the plasmid, the full-length *grt1*$^+$ was fused with Myc8his6 at the C terminus. The resulting plasmid (*pHYE37*) was linearized and used for transformation of a wild-type strain. Stable Leu+ transformants were selected and the linkage between the Leu+ of the transformants and Ura+ of the *grt1*$^+$ disruptant was confirmed.

Preparation of cell extracts, immunoprecipitation and immunoblotting

Cells were disrupted by vortexing in a modified HB buffer in the presence of glass beads (40 seconds, 4 times) and centrifuged at 5,000 rpm (3,500 ***g***) for 5 minutes. The resulting supernatants were used as

Table 1. Strains used in this study

Name	Genotype
SP39	*h+ leu1-32 cdc10-129*
SP1189	*h– leu1-32 cdc17-K42*
SP628	*h– leu1-32 cdc25-22*
SP1050	*h– leu1-32 nuc2-663*
SP1049	*h– leu1-32 cut9-665*
SP1047	*h– leu1-32 cut4-533*
AE202	*h– leu1-32 slp1-362*
AE166	*h– leu1-32 cut7-446*
AE204	*h– leu1-32 mts3-1*
SP858	*h+ leu1-32 nda3-KM311*
AE338	*h+ leu1-32 nda3-KM311 cut9+::cut9+-HA-LEU2*
HY81	*h– leu1-32 cut9-665 cdc25-22*
HY91	*h– leu1-32 mts3-1 cdc25-22*
HY128	*h– leu1-32 ura4-D18 grt1+::ura4 ade6-216*
HY137	*h– leu1-32 slp1-362 cut9+::cut9+-HA-LEU2*
HY141	*h– leu1-32 grt1+:: grt1+-Myc-Leu2*
HY148	*h– leu1-32 cdc25-22 grt1+:: grt1+-Myc-LEU2*
HY156	*h– leu1-32 nda3-KM311 grt1+:: grt1+-Myc-LEU2*
AE357	*h+/h– ade6-210/ade6-216 ura4-D18/ura4-D18 leu1-32/leu1-32*
AE295*	*h90 slp1-362 ste9::ura4 leu1-32 ura4-D18*
HY188	*h+ nda3-KM311 leu1-32 grt1+::grt1+-Myc-LEU2 cut9+::cut9+-HA-LEU2*

*AE295 is a generous gift from Dr Kenji Kitamura. Other strains were constructed in this study by crossing strains provided by Drs Mitsuhiro Yanagida and David Beach.

cell extracts for western blot. For immunoprecipitation, centrifugation at 14,000 rpm (10,000 ***g***) for 20 minutes was added and the supernatants were used. The modified HB buffer used in this study contained 25 mM Tris-HCl, pH 7.5, 15 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 0.5 mM Na_3VO_4, 10 mM N-ethylmaleimide (NEM, Sigma), 0.1% NP-40, 0.1 mM NaF and protease/proteasome inhibitors (1 mM PMSF, 2 μg/ml Aprotinin, 1 μg/ml Leupeptin, 10 μg/ml Trypsin Inhibitor, 10 μg/ml TPCK, 10 μM MG132 (CalBiochem)). Immunoprecipitation was performed following the procedures described by Stone et al. (1993). For SDS-PAGE, protein extracts were loaded at 50-150 μg/lane.

Cytological techniques

The procedures described by Hagan and Hyams (1988) were followed. For myc-tagged Grt1 immunofluorescence, cells were fixed by paraformaldehyde and glutaraldehyde (Sigma). Methanol fixation gave essentially the same result (not shown). An antibody to c-myc epitope (Boehringer Mannheim) was dissolved at a concentration of 0.4 mg/ml in PBS containing 0.1% NaN_3. The antibody solution was used at 100-300 times dilution. The secondary antibody, CY3-conjugated anti-mouse IgG, was used after diluting 1000-2000 times (Chemicon, 1 mg/ml stock solution).

Mad2 antibody

A part of the fission yeast *mad2*$^+$ (corresponding to amino acid position 28 to the C terminus) (He et al., 1997) was amplified by PCR using a pair of oligonucleotide primers, OL-12 (5′-GGC-AAGCTTCATATGGGATCCTACTACTAAGGATTCACTCGATA-3′) and OL-13 (5′-CGATCGATCATATGAACTCAATCCTTTTTCAA-CG-3′), and a *Zap*II-based fission yeast cDNA library as a template. A 0.5 kb *Nde*I fragment obtained by PCR was ligated into *pRK171A*. The resulting plasmid *pHYE17* was used to transform BL21(DE3). Bacterially expressed Mad2 was recovered as inclusion bodies. After boiling in SDS-PAGE sample buffer for 5 minutes, the sample was subjected to SDS-PAGE. A major band, p22, was eluted from the gel, and was used as the antigen to raise an anti-Mad2 antibody (Covance). The polyclonal rabbit crude antiserum was affinity-purified by the filter-binding method (Towbin et al., 1979).

RESULTS

Slp1 oscillates throughout the cell cycle

Slp1 is thought to activate APC and promote the selective proteolysis, which is an irreversible event and could be deleterious if it occurred inappropriately. To avoid this problem, the level of Slp1 may be strictly regulated in a cell cycle-dependent manner. We tested this hypothesis by measuring the Slp1 protein level at various cell cycle stages. Temperature-sensitive *cdc25-22* mutant cells were first arrested at the boundary of G_2/mitosis at the restrictive temperature (36°C) and then released to the permissive temperature (26°C). Following the shift to the permissive temperature, the cell cycle proceeds highly synchronously (Alfa et al., 1993). As shown in Fig. 1A, the level of the Slp1 protein reached a peak 40 minutes after the release and remained detectable for the next 40 minutes. Judging from the spindle index and cell plate index (Fig. 1C), we concluded that the Slp1 protein is detectable in a middle stage of mitosis. Oscillation of the protein level was accompanied by that of the transcript (Fig. 1B). The peaks of the RNA level appear slightly ahead of those of the protein level.

Sudden decrease in the protein level after the onset of anaphase prompted us to examine whether the Slp1 protein is degraded in an APC-dependent manner. Cell extracts were prepared from various cell cycle mutants and the Slp1 protein level was measured by western blot. As shown in Fig. 2A, the Slp1 protein accumulates in mutants arrested in mitosis. Most strikingly, it appeared highly stable in mutants defective in the anaphase selective proteolysis, suggesting that degradation of Slp1 is dependent on an active APC and 26S proteasome. Putative functional homologs of Slp1 are CDC20 in budding yeast and p55CDC in higher eukaryotes. It has been reported that the levels of these proteins behave similarly (Shirayama et al., 1998; Printz et al., 1998; Weinstein 1997).

It should be noted that the transcript is detectable even in interphase while the protein is only detectable in mitosis (Fig. 1A,B). This would suggest that the protein is rapidly degraded in interphase. If so, the Slp1 protein may be more stable in interphase in a mutant defective in proteolysis. Cell cycle progression was therefore arrested at interphase in various genetic backgrounds and cell extracts were prepared for western blot. In the *cdc25* single mutant arrested in interphase, the Slp1 protein is not detectable (Fig. 2B). Under these conditions, the *slp1*$^+$ gene is transcribed as shown in Fig. 1B (0 minutes after the shift). The amount of Slp1 scarcely increases in *cdc25 cut9* and *cdc25 mts3* double mutant cells (Fig. 2B), most of which are arrested in a late interphase due to the *cdc25* mutation, suggesting that the Slp1 is not degraded in interphase in an APC- or 26S proteasome-dependent manner.

Defects of ts *slp1-362*

A ts *slp1* (*slp1-362*) has been isolated previously and shown to be defective in transition from metaphase to anaphase (Matsumoto, 1997). In this study, we further characterized the defects of this allele. First, the mutant gene was cloned and its nucleotide sequence was compared to that of the wild-type gene. As shown in Fig. 3A, a single nonsense mutation was found at the 376th codon. The mutant gene encodes a truncated Slp1 protein that lacks two of the seven WD40 repeats. As a result, the molecular mass of the mutant Slp1 is reduced to approximately 43 kDa (Fig. 3B).

The wild-type Slp1 protein is readily detectable in mitosis, but not in interphase (Fig. 1A). When western blots were performed with cell extracts prepared from an asynchronous culture of a wild-type strain, the band corresponding to the wild-type Slp1 (55 kDa) appears very faintly (Fig. 3B). On the other hand, the truncated Slp1 encoded by *slp1-362* is easily detectable. The portion of the Slp1 protein missing in the *slp1* mutant may play an important role for regulation of its level throughout the cell cycle (see Discussion).

We found that the *slp1-362* mutant is lethal at the permissive temperature (26°C) when combined with a mutant that is arrested at metaphase due to a defect in proteolysis (*cut9*, *cut4*, *mts2* and *mts3*: Samejima and Yanagida, 1994; Yamashita et al., 1996; Gordon et al., 1993; Gordon et al., 1996). In contrast, it can grow at the permissive temperature in the background of *nda3* or *cut7*, which are arrested in metaphase due to a defective spindle formation (Hiraoka et al., 1984; Hagan and Yanagida, 1992). The specific genetic interaction would suggest that the defect in the onset of anaphase seen in the *slp1-362* mutant may directly result from a failure in physical interaction between the mutant Slp1 protein and a component necessary for the proteolysis. To test this possibility, we

performed immunoprecipitation and examined the binding ability of the Slp1 protein to APC. In order to prepare cell extracts containing a detectable amount of Slp1, cells were arrested in metaphase. In strains used for these experiments, Cut9, one of the subunits of APC, was tagged with HA epitope (Yamada et al., 1997), which can be recognized by a specific antibody. As shown in Fig. 4A, when cell extracts were prepared from *nda3-KM311*, a cold sensitive β-tubulin mutant, the anti-HA antibody precipitated HA-Cut9 as well as the wild-type Slp1 protein, suggesting that APC and the Slp1 protein physically interact with each other. Consistent with this result, the anti-Slp1 antibody precipitated both the Slp1 protein and HA-Cut9 from wild-type cell extracts. On the other hand, if cell extracts were prepared from the *slp1-362* mutant, the anti-HA antibody precipitated the HA-Cut9, but failed to precipitate the mutant Slp1 protein. Likewise, the anti-Slp1 antibody precipitated the mutant Slp1 protein, but not HA-Cut9. We also tested if the mutant Slp1 interacted with Cut9 in the background of *nda3-KM311* (Fig. 4B). While the anti-HA antibody precipitated the HA-Cut9, it did not precipitate the mutant Slp1 protein from the cell extracts prepared from a double mutant, *nda3-KM311 slp1-362*. Similarly, the anti-Slp1 antibody did not precipitate HA-Cut9.

As previously demonstrated, Cut9 is hyperphosphorylated in mitosis (Yamada et al., 1997). We noticed that the anti-Slp1 antibody preferentially precipitates the hyperphosphorylated form of HA-Cut9 from the wild-type cell extracts (Fig. 4). Thus, the majority of Slp1 bind to APC that contain the hyperphosphorylated Cut9. The phosphorylation of Cut9 somehow occurs to a lesser extent in *slp1-362* (Fig. 4A,B).

Isolation and disruption of *grt1*$^+$

In order to isolate any component with a functional link with Slp1 or a related component, we screened a fission yeast genomic library for a high dosage suppressor of *slp1-362*. One of the clones was found to suppress the *slp1* mutant at 32°C (Fig. 5A). The suppression at 36°C was partial. As shown in Fig. 5A, the transformants formed small colonies in which many cells were not viable. The clone was further subcloned into a *Hin*dIII 4 kb fragment and its nucleotide sequence was determined. Unidirectional deletion analysis from either end of the *Hin*dIII 4 kb fragment indicated that an approximately 2.8 kb region is required for suppression (Fig. 5B). The region contains a single open reading frame that would encode a protein with a predicted molecular mass of 74.3 kDa. We also confirmed that disruption of this open reading frame abolishes the suppressor activity (not shown). The open reading frame responsible for the suppression was designated *grt1*$^+$ (A cup of **gr**een **t**ea wakes up the sleepy guy. Accession Number for GenBank; AF236387). Overexpression of *grt1*$^+$ did not

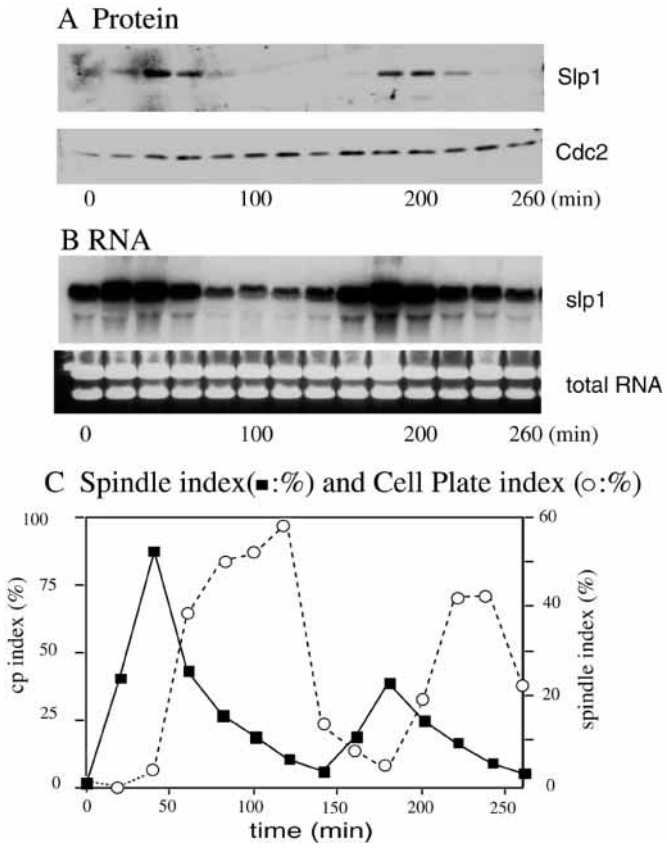

Fig. 1. Oscillation of Slp1 in the cell cycle. (A) Cell extracts prepared from a synchronous culture were analyzed by western blot with an antibody to Slp1 (upper panel). The same extracts were also analyzed with an antibody to Cdc2 to confirm that similar amounts of total proteins were loaded on each lane (lower panel). (B) Northern blot analysis of total RNA prepared from the culture used in the above experiment. Full-length cDNA of *slp1+* was used as a probe (upper panel). The lower panel shows an ethidium bromide-stained gel, indicating a similar amount of total RNA loaded in each lane. (C) Spindle and cell plate index of the synchronous culture at each time point after the release from the cdc25 block.

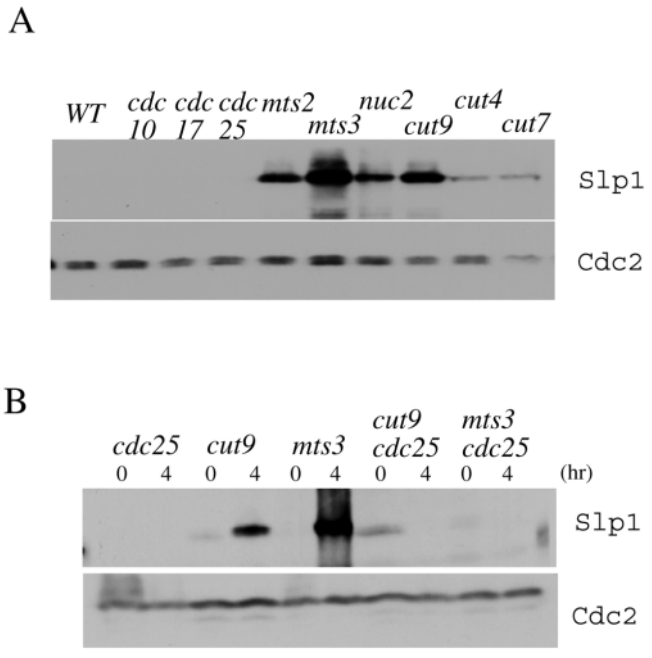

Fig. 2. Slp1 protein in cell cycle mutants. (A) Cell extracts were analyzed by western blot with either an antibody to Slp1 (upper panel) or to Cdc2 (lower panel). The genotype of each strain is indicated above the lane. Strains were incubated for 4 hours at the restrictive temperature, 36°C. (B) An experiment similar to A. Cell extracts were prepared before (0 hours) and 4 hours after the shift to the restrictive temperature.

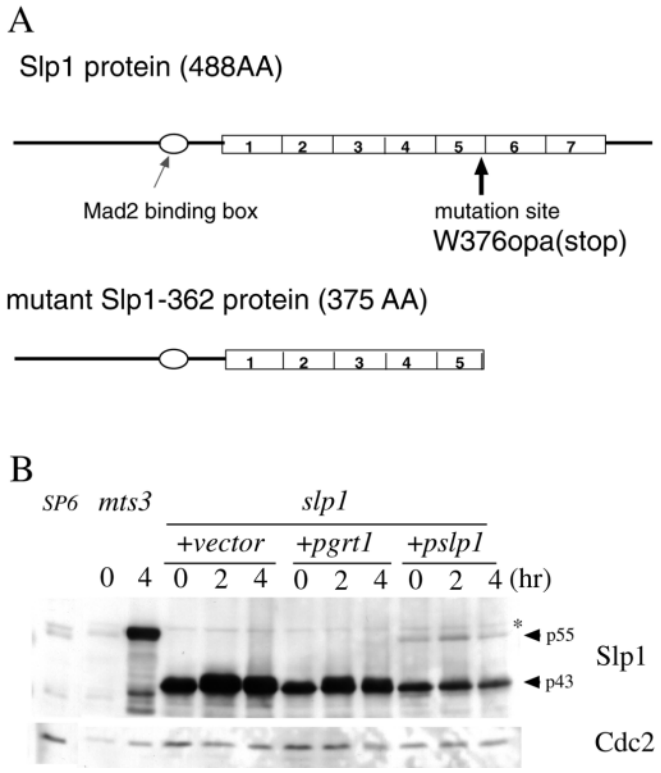

Fig. 3. A truncated Slp1-362 protein. (A) A schematic diagram of the structure of the Slp1 protein. Each numbered box represents a WD (tryptophan-aspartic acid) repeat motif. (B) Western blot was performed with cell extracts prepared from a wild-type strain (SP6), a *mts3-1* mutant before (0 hours) or 4 hours after the shift to the restrictive temperature, or *slp1-362* mutants, transformed with the indicated plasmids, that were incubated at the restrictive temperature for the indicated period (2 hours, 4 hours). The antibody to Slp1 was used as a probe. An asterisk indicates the position of a protein that weakly crossreacts with the antibody. The level of Cdc2 was also determined to confirm that similar amounts of total proteins were loaded on each lane.

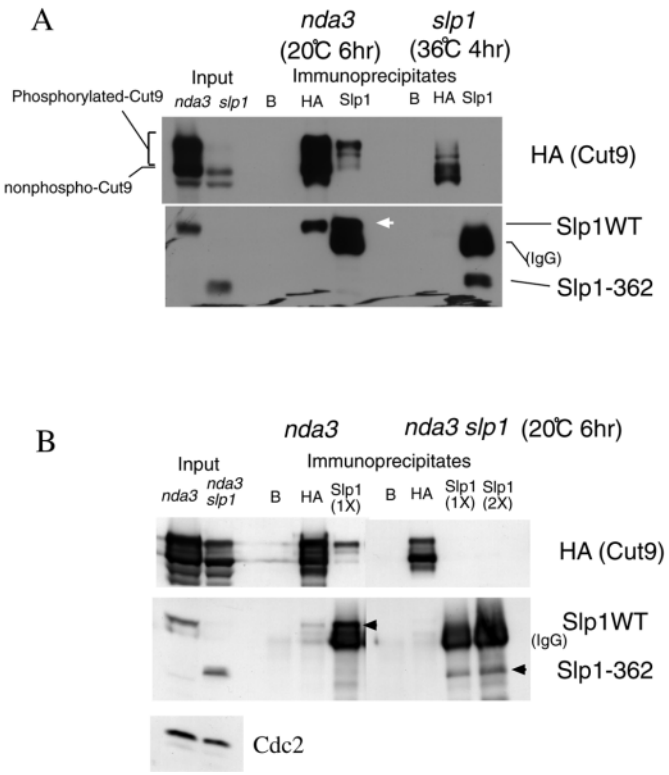

Fig. 4. Slp1-APC complex in wild type and in an *slp1-362* background. (A) Cell extracts were prepared after the shift to the restrictive temperature for each mutant (*nda3-KM311* or *slp1-362*) and used for immunoprecipitation with the antibody to the HA-epitope (HA) or the antibody to Slp1 (Slp1). A negative control experiment was performed with the protein A agarose beads alone without antibody (B). The extracts were also analyzed by western blot (Input). The immmunoprecipitates were subjected to SDS-PAGE and analyzed by western blot for the presence of HA-Cut9 (upper panel) and Slp1 (lower panel). The white arrow indicates the position of the wild-type Slp1 protein, which is very close to that of IgG. (B) A similar experiment was performed with cell extracts prepared from a *nda3-KM311* mutant or a double mutant, *nda3-KM311 slp1-362*. The black arrows indicate the position of the wild-type Slp1 and that of the mutant protein. Because the anti-Slp1 antibody (1×) precipitated the mutant protein less efficiently, the immunoprecipitation was also performed with increased amounts of the antibody (2×). Under these conditions HA-Cut9 was not detectable. The level of Cdc2 was also determined to confirm that similar amounts of total proteins were used in each experiment.

suppress Δ*slp1*, suggesting that *grt1*$^+$ does not bypass the function of Slp1.

Because the *slp1-362* allele encodes a truncated protein due to a nonsense mutation, it was possible that the suppression by *grt1*$^+$ might be due to a facilitated read-through of the nonsense mutation. To test this possibility, we examined the level of p43 protein (truncated protein coded by the mutant allele) and p55 protein (a full-length product from the read-through) in cell extracts prepared from the *slp1-362* mutant expressing *grt1*$^+$ at a high level. As shown in Fig. 3B, the p55 protein was detectable only in the *slp1-362* mutant transformed by the wild-type *slp1*$^+$ gene. High dosage expression of *grt1*$^+$ did not result in synthesis of p55 at a detectable level. The analysis also indicated that high dosage expression of *grt1*$^+$ did not increase the level of p43. Therefore, *grt1*$^+$ suppresses *slp1-362*, depending on a post-translational mechanism.

A database search indicated that the putative amino acid sequence of Grt1 contains a zinc finger motif (Evans and Hollenberg, 1988; Mackay and Crossley, 1998), at the N-terminal domain (Fig. 5C). Any known proteins in the database did not show significant homology to Grt1 except in the zinc finger domain. One copy of the *grt1*$^+$ gene was disrupted in a diploid by insertion of the *ura4*$^+$ gene. The diploid produced four viable segregants (two Ura+ and two Ura–), implying that the *grt1*$^+$ gene is not essential for growth. We further examined the *grt1*$^+$ disruptant (Δ*grt1*) phenotype. DAPI staining did not reveal any apparent abnormalities in nuclear morphology or cell shape. The strain was not notably sensitive to challenges with ultraviolet light (UV), Thiabendazole (TBZ), Hydroxyurea (HU), caffeine, canavanine and heavy metal (Cd^{2+}) (not shown). The minichromosome loss rate was comparable to that of the control strain. The double mutant of *slp1-362* Δ*grt1* did not show any apparent difference when compared to the single *slp1-362* mutant.

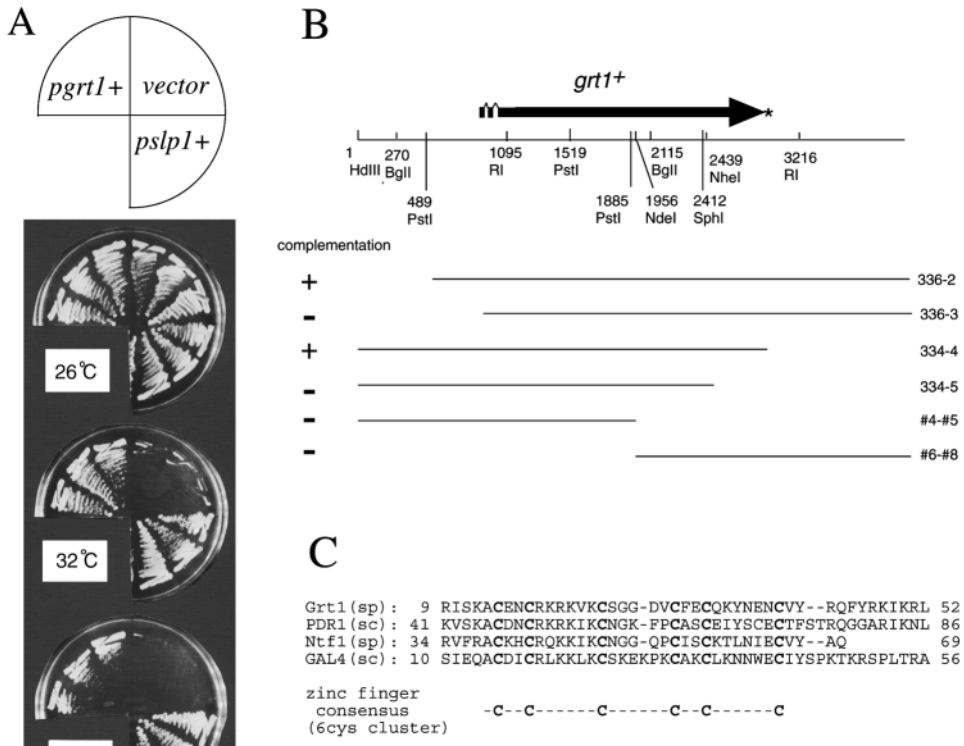

Fig. 5. High dosage *slp1* suppressor, *grt1*$^+$. (A) *slp1-362* was transformed with the indicated plasmid and incubated at the indicated temperature. (B) Strategy of subcloning to identify the active region. (C) Alignment of the zinc finger motif of Grt1 with that of other yeast proteins, PDR1 (Balzi et al., 1987), Ntf1 (Tang et al., 1994) and GAL4 (Laughon and Gesteland, 1984).

Genetic interaction of *grt1*$^+$

During this study, we found that Δ*ste9/srw1*$^+$ (Yamaguchi et al., 1997; Kitamura et al., 1998) partially suppresses *slp1-362* (Table 2). Thus, we tested whether the suppression by overexpression of *grt1*$^+$ is dependent on the *ste9/srw1*$^+$ function. A double mutant, *slp1-362* Δ*ste9*, was transformed with a plasmid for overexpression of *grt1*$^+$ and the growth ability of the transformants were examined at various temperatures. As summarized in Table 2, overexpression of *grt1*$^+$ raised the permissive temperature of the double mutant, *slp1-362* Δ*ste9*, indicating that *grt1*$^+$ has an additive effect. *grt1*$^+$ would function independently from *ste9*$^+$.

Similarly, we tested whether the suppression of *grt1*$^+$ is dependent on the wee1 function because *slp1-362* is partially suppressed by *wee1-50* (Matsumoto, 1997). A double mutant, *slp1-362 wee1-50*, could grow at a higher temperature when *grt1*$^+$ was overexpressed (Table 2), which would imply that *grt1*$^+$ functions independently from *wee1*$^+$.

We also tested whether overexpression of *grt1*$^+$ could cause a phenotype. First, it was possible that overexpression of *grt1*$^+$ could cause an effect similar to that of the *wee1-50* mutation. However, we did not find any apparent phenotypes induced by *grt1*$^+$ in the wild-type or *wee1-50* strains. Secondly, we suspected that *grt1*$^+$ could abrogate the spindle checkpoint, which would be a negative constraint for Slp1. The wild-type strain, however, did not show any elevated sensitivity to TBZ when overexpressing *grt1*$^+$. *slp1-362* was suppressed by deletion of *mad2*$^+$, to a much less extent than that by overexpression of *grt1*$^+$ (T. Matsumoto, unpublished result). These results indicate that the removal of the inhibitory effect on Slp1/APC is not sufficient to rescue *slp1-362*, and thus we speculate that *grt1*$^+$ function is involved mainly in facilitating anaphase progression, rather than in inhibition of the spindle checkpoint.

Grt1 protein

We examined the expression of the Grt1 protein by western blot. The predicted molecular mass of the HA-tagged Grt1 is about 79 kDa, and that of the Myc-tagged Grt1 about 86 kDa. As shown in Fig. 6A (lane 1), the Grt1 protein tagged with the HA epitope was detected as a band at 79 kDa in a wild-type strain. In addition, a smear could be detected at a higher molecular mass close to the origin as well as lower, around 43 kDa. Because (1) the band at 79 kDa and the smear could not be seen in a strain expressing no *grt1*$^+$ tagged with the HA-epitope (Fig. 6A, lane 9) and (2) a similar result was obtained with the Grt1 protein tagged with the Myc-epitope (Fig. 6B), we concluded that both the 79 kDa band and the smear represent the expressed Grt1 proteins. The smear would suggest that the Grt1 protein is highly modified. We examined whether the modification occurs in a manner dependent on the cell cycle. Cell extracts were prepared from a culture synchronized by the *cdc25* block and release method and were subjected to western blot. As shown in Fig. 6B, the amount of the protein, as well as the modification, did not change significantly throughout the cell cycle. We did, however, notice that the Grt1 protein was not detectable in the *nuc2-663* and *slp1-362* mutant arrested at metaphase (Fig. 6A, lanes 6,7). Since cell extracts prepared from other mutants arrested at metaphase or anaphase contained the Grt1 protein at a detectable level (Fig. 6A, lanes 2-5,8), the reduction is specific to the two mutants. We repeated the experiments and consistently found that the level of the Grt1 protein was lower

Table 2. Growth of *slp1-362*

Genotype	Plasmid	Temperature (°C) 26	30.5	32	36
Wild type		+++	+++	+++	+++
slp1-362		++	++	–	–
slp1-362 ste9+::ura4		++	++	+	–
slp1-362 wee1-50		++	++	+	–
slp1-362	*grt1*$^+$	++	++	++	±
slp1-362 ste9+::ura4	*grt1*$^+$	++	++	++	+
slp1-362 wee1-50	*grt1*$^+$	++	++	++	+

Growth rate of each strain was summarized.

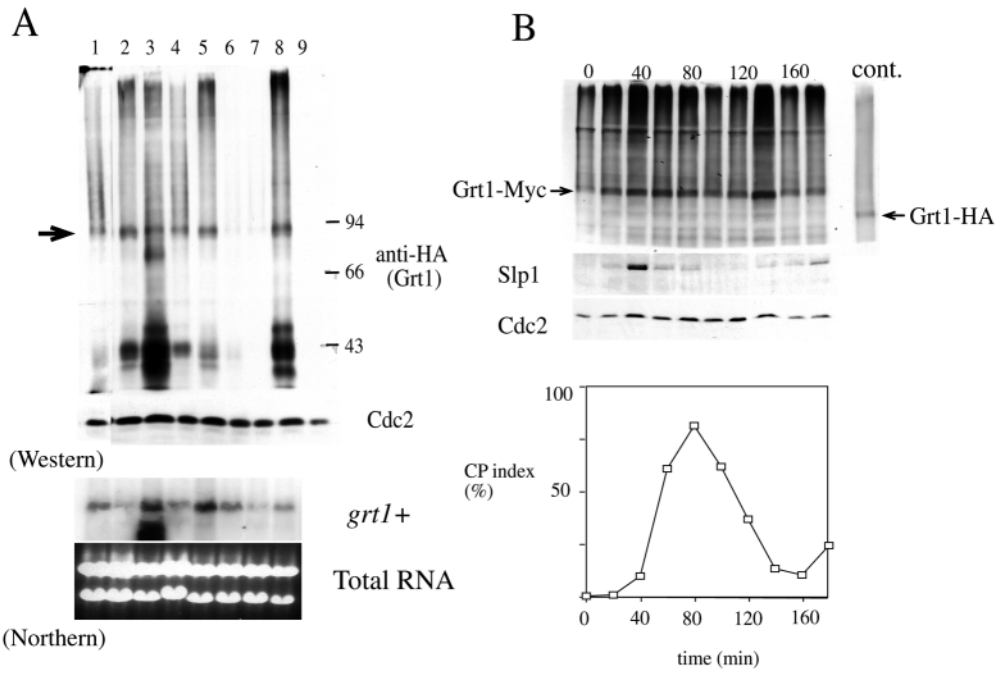

Fig. 6. Grt1 protein. (A) Cell extracts were prepared from the following strains expressing HA-tagged Grt1: wild type (lane 1), *cdc25-22* (lane 2), *nda3-KM311* (lane 3), *cut4-533* (lane 4), *cut9-665* (lane 5), *nuc2-663* (lane 6), *slp1-362* (lane 7) and *mts3-1* (lane 8). As a negative control, extracts were also prepared from a strain expressing no HA-tagged Grt1 (lane 9). Western blotting was performed with either the antibody to the HA epitope to detect HA-Grt1 or the antibody to Cdc2 to confirm that a similar amount of total proteins was loaded on each lane. Northern blottingwas performed with total RNAs prepared from the above strains, omitting the strain expressing no HA-tagged Grt1 (lane 9). The result is shown in the order same as the above western blot. The *grt1*$^+$ cDNA was used as a probe. (B) Cell extracts were prepared from a synchronous culture of a *cdc25* mutant expressing Myc-tagged Grt1 (single copy integrant) and analyzed by western blot with either the antibody to Myc epitope (upper panel), the antibody to Slp1 (middle panel) or the antibody to Cdc2 (lower panel). Time after the release from the *cdc25* block is indicated above the panels. Extracts were also prepared from a strain expressing HA-tagged Grt1 and analyzed by the antibody to HA epitope. Note that the bands of the Myc-tagged Grt1 appear at a position of higher molecular mass than that of HA-tagged Grt1. This is because tagging with Myc epitope results in a more increase in the molecular mass. It should also be noted that the culture was highly synchronized, as indicated by sharp peaks seen at the level of Slp1 and the cell plate (CP) index.

in the two mutants. In order to determine the level of the transcript of *grt1*$^+$ in each mutant, northern blots were performed. Total RNA from a wild-type strain (lane 1), a *cut4* mutant (lane 4) and a *nuc2* mutant (lane 6) contained the *grt1*$^+$ transcript at similar levels, whereas the level of the Grt1 protein in the *nuc2* mutant was significantly lower than the other two strains. Similarly, both a *cdc25* mutant and a *slp1* mutant expressed comparable amounts of the transcript of *grt1*$^+$, the level of the Grt1 protein being much lower in the *slp1* mutant. Therefore, analysis of the transcript indicated that the reduction in the level of the Grt1 protein in the *slp1* and *nuc2* mutants is not due to a reduction in the transcript.

Finally, we tested whether Grt1 protein could physically interact with Slp1 or proteins that interacted with Slp1. Cell extracts were prepared from a *nda3-KM311* mutant expressing Cut9 tagged with HA epitope and Grt1 tagged with Myc epitope. The strain was incubated at the restrictive temperature (20°C) for 6 hours and thus arrested at metaphase due to a defective spindle formation. Under these conditions Slp1 was at a detectable level and Cut9 was hyperphosphorylated (Fig. 7A, Input). Immunoprecipitates with the anti-HA antibody contained HA-Cut9 as well as Slp1. The precipitates also contained Mad2, a component of the spindle checkpoint. This result is consistent with previous studies in mammalian cells (Fang et al., 1998; Kallio et al., 1998), which demonstrated the ternary complex of Mad2-APC-p55CDC (a mammalian homolog Slp1). The precipitates, however, did not contain Myc-tagged Grt1. Likewise the precipitates with the anti-Myc antibody did not contain Slp1, HA-Cut9 or Mad2. The other two antibodies, anti-Slp1 antibody and anti-Mad2 antibody, could precipitate the ternary complex, Mad2-APC-Slp1, but failed to precipitate Myc-tagged Grt1. These results indicated that Grt1 did not tightly associate with Slp1, Mad2 or APC under these experimental conditions. We also performed a similar experiment in an *mts3*-arrest condition and did not observe Grt1 in the Slp1-APC complex. Other experiments to test the physical interaction of Grt1 with Cut2, or a 26S proteasome component Mts4 (Wilkinson et al., 1997), showed no tight association between Grt1 and these components (data not shown).

Cell extracts prepared from the *nda3* mutant were also analyzed by a sucrose gradient centrifugation as described previously (Yamada et al., 1997). As shown in Fig. 7B, HA tagged-Cut9 broadly distributed through the fractions with a peak at fractions 10 and 11. The peak contained the hyperphosphorylated form of Cut9 and is thought to represent the functional APC (Yamada et al., 1997). In the same gradient, Myc-tagged Grt1 showed a peak around fractions 4-7, suggesting that Grt1 does not interact with functional APC.

DISCUSSION

Oscillation of Slp1

Like most of the CDC20 homologs in other organisms (Shirayama et al., 1998; Printz et al., 1998; Weinstein et al., 1997), the level of the Slp1 protein oscillates throughout the cell cycle. The protein level seems to be regulated by multiple mechanisms. Firstly, the change in the transcript largely contributes to that of the protein. Secondly, the sharp decrease

in late mitosis requires the active APC and the 26S proteasome. Therefore, Slp1 is possibly a substrate for the selective proteolysis at anaphase. It has been shown that CDC20, a budding yeast homolog of Slp1, contains two destruction boxes at the N terminus and that they are necessary for the proteolysis (Shirayama et al., 1998). Despite our search, we could not identify a motif typical of the destruction box (Yamano et al., 1998) nor the KEN box, a newly identified destruction signal sequence (Pfleger and Kirschner, 2000) in the amino acid sequence of Slp1. It is possible that the Slp1 protein may contain a less conserved motif of the destruction box. Finally, we have noticed that while the transcript is readily detectable throughout the cell cycle, the protein is hardly detectable in interphase. The Slp1 may be rapidly destructed in interphase in a manner independent on APC/proteasome (Fig. 2B), or the translation of the Slp1 protein is tightly regulated. Through multiple levels of control mechanisms, the amount of Slp1 is strictly regulated in such a way that the protein exists for only a limited period of the cell cycle, mitosis. Regulation of the level of Slp1 protein is not only the mechanism controlling the activity of Slp1, however; we have previously shown that Slp1 is a target of the spindle checkpoint. Mad2, a component of the checkpoint, binds to Slp1 and inhibits its activity (Kim et al., 1998).

Defects of *slp1-362*

We speculate, by analogy with budding yeast CDC20, that Slp1 promotes the APC function. As shown in Fig. 4, the wild-type Slp1 (p55) can physically interact with APC. The Slp1-362 mutant protein (p43) cannot form a complex with APC when arrested at the restrictive temperature for the *slp1-362* mutant or at the restrictive temperature for the *nda3-KM311* mutant, which would suggest that APC activation function in the mutant is impaired. The truncated Slp1 is highly stable in cells growing at the permissive temperature (26°C) and its level slightly increases at the restrictive temperature (36°C). We speculate that interaction of Slp1 with APC normally leads to ubiquitination of Slp1 followed by degradation. In contrast, the Slp1-362 mutant protein, which is less competent to interact with APC, would not be properly ubiquitinated and remains stable. The last 113 amino acids missing in the mutant may play an important role in direct physical interaction with APC.

During our analysis of the complex formation of Slp1-APC, we noticed that (1) the wild-type Slp1 protein preferentially associates with the hyperphosphorylated Cut9 and (2) this phosphorylation occurs to a lesser extent in the *slp1-362* mutant. Association of Slp1 to APC may induce the phosphorylation of Cut9. It is also possible that Slp1 positively regulates a kinase that is responsible for the phosphorylation of Cut9, which would suggest that the mutant Slp1 protein is defective in regulation of the kinase and thus cannot produce the phosphorylated Cut9. It has been reported that p55CDC, a mammalian homolog of Slp1, associates with a kinase (Weinstein et al., 1994).

In this study, genetic analysis has revealed that *slp1-362* is partially suppressed by deletion of *ste9/srw1*$^{+}$. Budding yeast

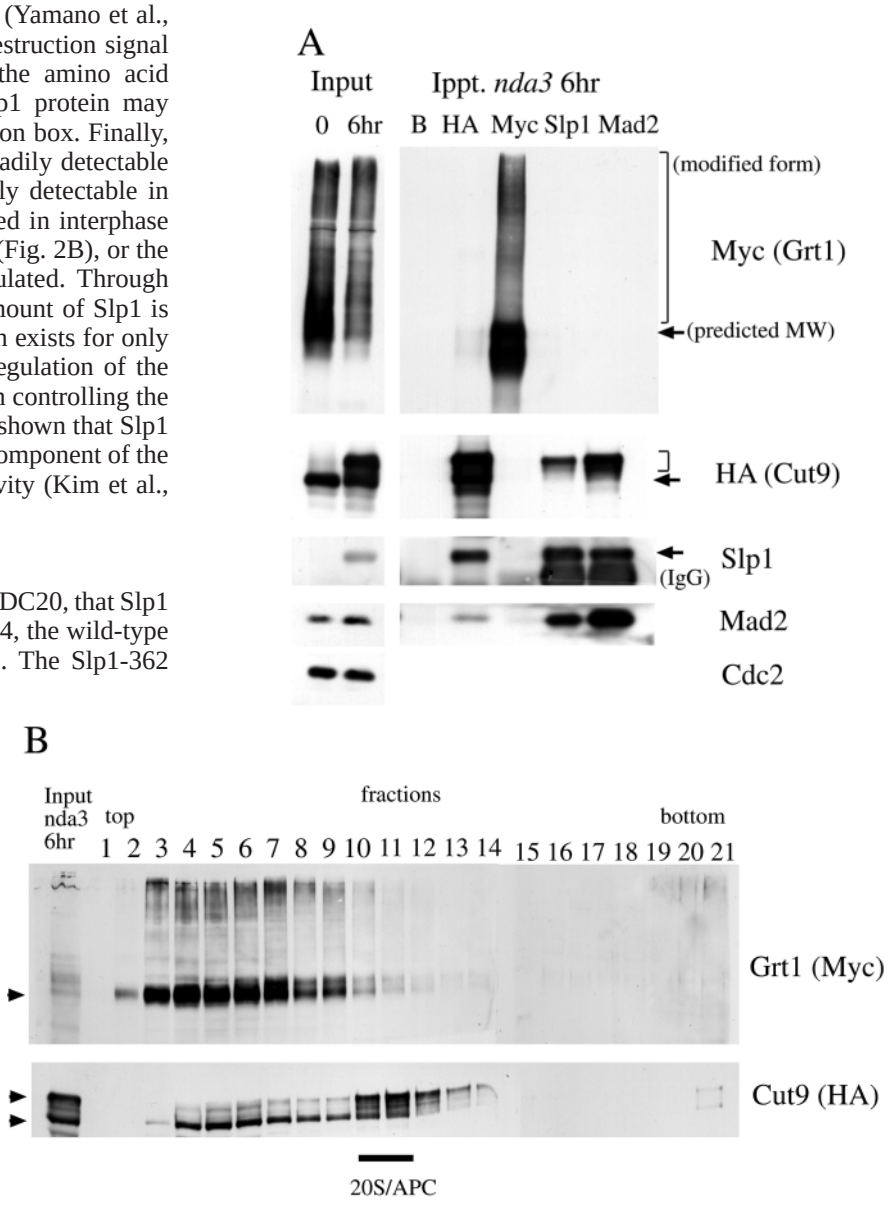

Fig. 7. Physical interaction between APC(Cut9), Mad2, Slp1 and Grt1. (A) Cell extracts were prepared from a *nda3-KM311* mutant that expressed HA-Cut9 and Myc-Grt1. Immunoprecipitation (Ippt) was performed with the antibodies indicated above each lane. A negative control experiment was performed with the protein A agarose beads alone and no antibody (lane B). Immunoprecipitates, subjected to SDS-PAGE, were examined for the presence of proteins, Grt1, Cut9, Slp1 or Mad2, by western blot using antibodies indicated on the right side of each panel. The level of Cdc2 was also determined to confirm that a similar amount of total proteins were used. Although there is a very faint band detected by the anti-Myc antibody in the lane of the precipitates by the anti-HA antibody, it would not be a sufficiently convincing result to suggest any interaction between HA-Cut9 and Myc-Grt1. The corresponding band cannot be detected in the reciprocal immunoprecipitation. (B) Cell extracts prepared from the *nda3* mutant were subjected to a sucrose gradient centrifugation and fractionated. Each fraction was test by western blot with the anti-Myc antibody for the presence of Grt1 (upper panel) or with the anti-HA antibody for the presence of Cut9 (lower panel).

Cdh1/Hct1, a putative homolog of Ste9/Srw1, binds to APC, and loses its affinity to APC when it is phosphorylated by the Cdc2 kinase. It has also been demonstrated that CDC20, a budding yeast homolog of Slp1, can bind to APC when Cdh1/Hct1 cannot, due to the phosphorylation (Zachariae et al., 1998b). CDC20 and Cdh1/Hct1 appear to compete for binding to APC. We propose that fission yeast Slp1 and Ste9/Srw1 have a similar relationship. Deletion of *ste9/srw1*$^+$ would make a favorable environment for Slp1-362 to bind to APC.

High dosage suppressor, *grt1*$^+$

Through a genetic screen for a high dosage suppressor of *slp1-362*, we have isolated *grt1*$^+$. The putative open reading frame of Grt1 contains a zinc finger motif. The alignment in Fig. 5C indicates not only that the six cysteine residues that form the core consensus motif, but also many of the internal amino acids, are conserved between Grt1 and other yeast zinc finger proteins. The motif has been identified in a number of transcriptional factors and has DNA-binding activity (Evans and Hollenberg, 1988; Balzi et al., 1987; Laughon and Gesteland, 1984; Tang et al., 1994). At present we do not have any evidence to suggest that there is DNA binding activity in Grt1 protein. We have found that Grt1 tagged with the HA epitope localizes in the nucleus, but not a particular domain of chromatin (this construct slightly reduces the suppression activity of *grt1*$^+$ and it is an artifactual condition). This preliminary result may suggest that Grt1 is not a DNA binding protein. A similar motif is also believed to play a role in interacting with proteins (Mackay and Crossley, 1998). As proposed for PKC and DGK (diacylglycerol kinase), chemical compounds such as phorbol ester and diacylglycerol may be able to interact with the motif (van Blitterswijk and Houssa, 1999). Another zinc-binding motif, RING finger, has been found in proteins which function in the ubiquitin-ligation system such as APC and SCF (Zachariae et al., 1998a; Kammura et al., 1999; Seol et al., 1999). Fission yeast Zfs1 protein, required to prevent septation if mitotic progression is inhibited, also contains two zinc fingers (Beltraminelli et al., 1999).

Grt1 protein

We found that the Grt1 protein appears highly modified on the conventional SDS-PAGE. A portion of the Grt1 protein tagged with the HA-epitope was detected as a relatively discrete band at 79 kDa, as predicted (Fig. 6). In addition, a significant amount of the protein was detected as a smear as well as diffused bands at higher and lower molecular masses. Our preliminary study has suggested that this modification may not be due to ubiquitination or phosphorylation. Intriguingly, it has been reported that Cut4, APC1 homolog and a component of fission yeast APC, is also heavily modified and appears similarly on conventional SDS-PAGE (Yamashita et al., 1996; Yamashita et al., 1999; Yanagida et al., 1999).

The most striking feature of Grt1 is its instability in *slp1-362*. While the protein was readily detectable in other mutants arrested in mitosis, it was hardly detectable in *slp1-362*. Northern blot analysis indicated that the reduction in the protein level was not due to the transcript. Most likely, Grt1 is unstable in *slp1-362*. In this study we have found two defects of the *slp1-362*, which are the inability (1) to interact with APC and (2) to maintain the normal level of Grt1. Because the high dosage expression of *grt1*$^+$ suppresses *slp1-362*, the second defect may represent the physiological condition of *slp1-362* more directly. Interestingly, the *nuc2-663* mutant also fails to maintain the Grt1 protein, though this mutant could not be suppressed by overexpression of *grt1*$^+$ (Yamada, unpublished result). It should be noted that the two mutants, *slp1-362* and *nuc2-663*, share some characters. Firstly, the *nuc2* mutant can be partially suppressed by overexpression of *slp1*$^+$ (H. Y. Yamada and M. Yanagida, unpublished result). Secondly, among a number of APC-defective mutants, these two mutants are arrested tightly at metaphase. As indicated by the locus name '*cut*', other APC-defective mutants such as *cut4* and *cut9* form a septum, which often goes across the unseparated nucleus. In contrast, *slp1-362* and *nuc2-663* maintain condensed chromosomes, but do not initiate cytokinesis. Loss of Grt1 might result in a tighter arrest at metaphase.

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REFERENCES

Alfa, C., Fantes, P., Hyams, J., McLeod, M. and Warbrick, E. (1993). *Experiments with Fission Yeast: A Laboratory Course Manual*, pp. 48-51. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Amon, A. (1999). The spindle checkpoint. *Curr. Opin. Genet. Dev.* **9**, 69-75.

Balzi, E., Chen, W., Ulaszewski, S., Capieaux, E. and Goffeau, A. (1987). The multidrug resistance gene PDR1 from *Saccharomyces cerevisiae. J. Biol. Chem.* **262**, 16871-16879.

Beltraminelli, N., Murone, M. and Simanis,V. (1999). The *S. pombe zfs1* gene is required to prevent septation if mitotic progression is inhibited. *J. Cell Sci.* **112**, 3103-3114.

Cohen-Fix, O., Peters, J., Kirschner, M. and Koshland, D. (1996). Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* **10**, 3081-3093.

Evans, R. M. and Hollenberg, S. M. (1988). Zinc fingers: Gilt by association. *Cell* **52**, 1-3.

Fang, G., Yu, H. and Kirschner, M. W. (1998). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase intitiation. *Genes Dev.* **12**, 1871-1883.

Funabiki, H., Kumada, K. and Yanagida, M. (1996a). Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle, and form large complexes. *EMBO J.* **15**, 6617-6628.

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

Funabiki, H., Yamano, H., Nagao, K., Tanaka, H., Yasuda, H., Hunt, T. and Yanagida, M. (1997). Fission yeast Cut2 required for anaphase has two destruction boxes. *EMBO J.* **16**, 5977-5987.

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

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

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.

Gordon, C., McGurk, G., Wallace, M. and Hastie, N. D. (1996). A conditional lethal mutant in the fission yeast 26S protease subunit *mts3*$^+$ is defective in metaphase to anaphase transition. *J. Biol. Chem.* **271**, 5704-5711.

Grimm, C., Kohli, J., Murray, J. and Maundrell, K. (1988). Genetic engineering of *Schizosaccharomyces pombe:* a system for gene disruption

and replacement using the ura4 gene as s selectable marker. *Mol. Gen. Genet.* **215**, 81-86.

Gutz, H., Heslot, H., Leupold, U. and Lopreno, N. (1974). *Schizosaccharomyces pombe*. In *Handbook of Genetics*, vol. 1 (ed. R. C. King), pp. 395-446. New York: Plenum Press.

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. (1992). Kinesin-related cut7 protein associates with mitotic and meiotic spindles in fission yeast. *Nature* **356**, 74-76.

Hardwick, K. G. (1998). The spindle checkpoint. *Trends Genet.* **14**, 1-4

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.

Hershko, A. (1997). Roles of ubiquitin-mediated proteolysis in cell cycle control. *Curr. Opin. Cell Biol.* **9**, 788-99.

Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R. E. and Cohen, L. H. (1991). Methylated ubiquitin inhibits cyclin degradation in clam embryo extracts. *J. Biol. Chem.* **266**, 16376-9

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

Hiraoka, Y., Toda, T. and Yanagida, M. (1984). The *NDA3* gene of fission yeast encodes b-tubulin: A cold sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell* **39**, 349-358.

Holloway, S. L., 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.

Hochstrasser, M. (1995). Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr. Opin. Cell Biol.* **7**, 215-223.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.* **30**, 405-439.

Hwang, L. H., Lau, L. F., Smith, D. L., Mistrot, C. A., Hardwick, K. G., Hwang, E. S., Amon, A. and Murray, A. W. (1998). Budding yeast Cdc20: A target of the spindle checkpoint. *Science* **279**, 1041-1044.

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.

Juang, Y.-L., Huang, J., Peters, J.-M., McLaughlin, M. E., Tai, C.-Y. and Pellman, D. (1997). APC-mediated proteolysis of Ase1 and the morphogenesis of the mitotic spindle. *Science* **275**, 1311-1314.

Kallio, M., Weinstein, J., Daum, J. R., Durk, D. J. and Gorbsky, G. J. (1998). Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex and is involved in regulating anaphase onset and late mitotic events. *J. Cell Biol.* **141**, 1393-1406.

Kamura, T., Conrad, M. N., Yan, Q., Conaway, R. C. and Conaway, J. W. (1999). The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. *Genes Dev.* **13**, 2928-33

Kim, S. H., Lin, D. P., Matsumoto, S., Kitazono, A. and Matsumoto, T. (1998). Fission yeast Slp1: An effector of the Mad2- dependent spindle checkpoint. *Science* **279**, 1045–1047.

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.

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.

Laughon, A. and Gesteland, R. F. (1984). Primary structure of the *Saccharomyces cerevisiae* GAL4 gene. *Mol. Cell Biol.* **4**, 260-267.

Mackay, J. P. and Crossley, M. (1998). Zinc fingers are sticking together. *Trends Biochem. Sci.* **23**, 1-4 .

Matsumoto, T. (1997). A fission yeast homolog of CDC20/p55CDC/Fizzy is required for recovery from DNA damage and genetically interacts with p34cdc2. *Mol. Cell. Biol.* **17**, 742-750.

Moreno, S., Klar, A. and Nurse, P. (1991). An introduction to molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. *Meth. Enzymol.* **56**, 795-823.

Morgan, D. O. (1999). Regulation of the APC and the exit from mitosis. *Nat. Cell Biol.* **1**, E47-53.

Murray, A. W., Solomon, M. J. and Kirschner, M. W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* **339**, 280-286.

Peters, J.-M. (1998). SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. *Curr. Opin. Cell Biol.* **10**, 759-768.

Pfleger, C. M. and Kirschner, M. W. (2000). The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev.* **14**, 655-665.

Prinz, S., Hwang, E. S. Visintin, R. and Amon, A. (1998). The regulation of Cdc20 proteolysis reveals a role for the APC components Cdc23 and Cdc27 during S phase and early mitosis. *Curr. Biol.* **8**, 750-760.

Samejima, I. and Yanagida, M. (1994). Bypassing anaphase by fission yeast *cut9* mutation: Requirement of *cut9*$^{+}$ gene to initiate anaphase. *J. Cell Biol.* **127**, 1665-1670.

Schwab, M., Lutum, A. S. and Saufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis *Cell* **90**, 683-693.

Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K., Shevchenko, A. and Deshaies, R. J. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes Dev.* **13**, 1614-26.

Sethi, N., Monteagudo, M. C., Koshland, D., Hogan, E. and Burke, D. (1991). The CDC20 Gene Product of *Saccharomyces cerevisiae*, a beta-Transducin Homolog, Is Required for a Subset of Microtuble-Dependent Cellular Processes. *Mol. Cell. Biol.* **11**, 5592-5602.

Shirayama, M., Zachariae, W., Ciosk, R. and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J.* **17**, 1336-1349.

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.

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.

Tang, C.S., Bueno, A. and Russell, P. (1994). *ntf1+* encodes a 6-cysteine zinc finger-containing transcription factor that regulates the nmt1 promoter in fission yeast. *J. Biol. Chem.* **269**, 11921-11926.

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

Townsley, F. M. and Ruderman, J. V. (1998). Proteolytic ratchets that control progression through mitosis. *Trends Cell Biol.* **8**, 238-244.

van Blitterswijk, W. J. and Houssa, B. (1999**)**. Diacylglycerol kinases in signal transduction. *Chem. Phys. Lipids* **98**, 95-108.

Visintin, R., Prinz, S. and Amon, A. (1997). CDC20 and CDH1: A family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**, 460-463.

Weinstein, J., Jacobsen, F.W., Hsu-Chen, J., Wu, T. and Baum, L.G. (1994). A novel mammalian protein, p55CDC, present in dividing cells is associated with protein kinase activity and has homology to the *Saccharomyces cerevisiae* cell division cycle proteins Cdc20 and Cdc4. *Mol. Cell. Biol.* **14**, 3350-3363.

Weinstein, J. (1997). Cell cycle-regulated expression, phosphorylation, and degradation of p55Cdc. A mammalian homolog of CDC20/Fizzy/slp1. *J. Biol. Chem.* **272**, 28501-28511.

Wilkinson, C. R., Wallace, M., Seeger, M., Dubiel, W. and Gordon, C. (1997). Mts4, a non-ATPase subunit of the 26 S protease in fission yeast is essential for mitosis and interacts directly with the ATPase subunit Mts2. *J. Biol. Chem.* **272**, 25768-25777.

Yamada, H., Kumada, K. and Yanagida, M. (1997). Distinct subunit functions and cell cycle regulated phosphorylation of 20S APC/cyclosome required for anaphase in fission yeast. *J. Cell Sci.* **110,** 1793-1804.

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.

Yamamoto, A., Guacci, V. and Koshland, D. (1996). Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *J. Cell Biol.* **133**, 99-110.

Yamano, H., Tsurumi, C., Gannon, J. and Hunt, T. (1998). The role of the destruction box and its neighbouring lysine residues in cyclin B for anaphase ubiquitin-dependent proteolysis in fission yeast: defining the D-box receptor. *EMBO J.* **17**, 5670-5678.

Yamashita, Y. M., Nakaseko, Y., Samejima, I., Kazuki, K., Yamada, H.,

Michaelson, D. and Yanagida, M. (1996). 20S cyclosome complex formation and proteolytic activity inhibited by the cAMP/PKA pathway. *Nature* **384**, 276-279.

Yamashita, Y. M., Nakaseko, Y., Kumada, K., Nakagawa, T. and Yanagida, M. (1999). Fission yeast APC/cyclosome subunits, Cut20/Apc4 and Cut23/Apc8, in regulating metaphase-anaphase progression and cellular stress responses. *Genes Cells* **4**, 445-463.

Yanagida, M. (1998). Fission yeast *cut* mutations revisited: control of anaphase. *Trends Cell Biol.* **4**,144-149.

Yanagida, M., Yamashita, Y.M., Tatebe, H., Ishii, K., Kumada, K. and Nakaseko, Y. (1999). Control of metaphase-anaphase progression by proteolysis: cyclosome function regulated by the protein kinase A pathway, ubiquitination and localization. *Phil. Trans. R. Soc. Lond.* B **354**, 1559-1569.

Zachariae, W. and Nasmyth, K. (1996). TPR proteins required for anaphase progression mediate ubiquitination of mitotic B-type cyclins in yeast. *Mol. Biol. Cell* **7**, 791-801.

Zachariae, W., Shin, T. H., Galova, M., Obermaier, B. and Nasmyth, K. (1996). Identification of subunits of the anaphase-promoting complex of *Saccharomyces cerevisiae*. *Science* **274**, 1201-1204.

Zachariae, W., Shevchenko, A., Andrews, P. D., Ciosk, R., Galova, M., Stark, M. J., Mann, M. and Nasmyth, K. (1998a). Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. *Science* **279**, 1216-1219.

Zachariae, W., Schwab, M., Nasmyth, K. and Saufert, W. (1998b). Control of Cyclin Ubiquitination by CDK-Regulated Binding of Hct1 to the Anaphase Promoting Complex. *Science* **282**, 1721-1724.

Zachariae, W. and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev.* **13**. 2039-2058.