the *nimA* gene normally arrest in S and G_2 phase, respectively, but enter mitosis if *bimE* has been mutated (*18*). It is presently unclear why mutation of an APC subunit would cause this phenotype. One possibility is that mitotic cyclins accumulate in abnormal amounts in *bimE* mutants, resulting in premature entrance into mitosis. Alternatively, APC may negatively regulate other activators of mitosis such as NIMA (*21*). Others have proposed that APC may also regulate exit from S phase (*22*). Because APC is composed of eight subunits, it is conceivable that APC ubiquitinates multiple proteins that regulate cell-cycle progression.

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9. S100 prepared from interphase or mitotic *Xenopus* egg extracts was fractionated by Resource Q chromatography as described (*5*), except that the column volume was 50 ml, the volume of the salt gradient was 300 ml, and the fraction size was 15 ml. APC eluted between 345 and 450 mM KCl (corresponding to fractions 13 through 15 in Fig. 2, A and B) and was bound to beads covalently coupled with antibodies to CDC27 (*5*). The beads were washed with 40 volumes of buffer containing 15 mM tris-HCl (pH 7.7), 1 mM $MgCl_2$, 475 mM KCl, and 0.5% NP40. Ubiquitination assays with APC bound to antibody beads and elution of APC from the beads for SDS–polyacrylamide gel electrophoresis (PAGE) analysis were done as described (*5*). APC preparations from two different mitotic extracts (Δ90 extracts and extracts from metaphase II–arrested eggs) (*5*) had identical subunit compositions and ubiquitination activities.
10. Mitotic APC (~16 μg) bound to antibody beads was incubated for 30 min at 30°C in 100 μl of buffer [50 mM tris-HCl (pH 7.8), 1 mM dithiothreitol, 2 mM $MnCl_2$, and 100 μg/ml bovine serum albumin (BSA)] containing either 33 μg λ-PPase (300 units per microgram; Biolabs) or no λ-PPase. BSA and phosphatase were removed by washing the beads as described (*9*).
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12. APC (~250 μg) was purified from 500 ml of interphase *Xenopus* extract (15 g of protein) (*9*). Individual polypeptides were separated by SDS-PAGE, transferred to polyvinyldifluoride membrane (Bio-Rad) and processed for microsequencing at the Harvard Microchemistry Facility as described (*6*). APC1 and APC7 were digested with Lys-C, and APC3 and APC6 were digested with trypsin. The following sequences were obtained: APC1, DYIAPLPFQVANVXP, AEEQNAVLNLDQLGTPQHGMTTSSLTANLR, LSWTRNCDFEGSL, and PMTSIG; APC3, LLHLPAALGPLNPQFGIL, ILFANEK, and ALQELEELK; APC6, EFDFER, CYDFDVHTMK, and GLXLTAQY; APC7, EFFLAHIYTELQLIEEAL. Only high confidence sequences are listed, except for the third APC1 sequence which is derived from an equimolar mixture of two peptides. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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17. Membranes were isolated as described (*5*) with one additional washing step, and S100 and Resource Q column fractions were prepared as in (*9*). Fractions 13 to 15 (Fig. 2B) were pooled and further separated on a Superose 6 HR 10/30 column (Pharmacia) equilibrated in buffer Q-A (*5*). APC eluted with a molecular mass of 1.6 million daltons.
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Identification of Subunits of the Anaphase-Promoting Complex of *Saccharomyces cerevisiae*

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Entry into anaphase and proteolysis of B-type cyclins depend on a complex containing the tetratricopeptide repeat proteins Cdc16p, Cdc23p, and Cdc27p. This particle, called the anaphase-promoting complex (APC) or cyclosome, functions as a cell cycle–regulated ubiquitin-protein ligase. Two additional subunits of the budding yeast APC were identified: The largest subunit, encoded by the *APC1* gene, is conserved between fungi and vertebrates and shows similarity to BIMEp from *Aspergillus nidulans*. A small heat-inducible subunit is encoded by the *CDC26* gene. The yeast APC is a 36*S* particle that contains at least seven different proteins.

Mitotic cyclin degradation is required for the final exit from mitosis (*1*, *2*) and is a prerequisite for S phase in the subsequent cell cycle (*3*). In extracts from *Xenopus* eggs, degradation of cyclin B depends on a particle called the APC, which contains at least eight different proteins. The APC and the cyclosome, a particle found in clam oocytes, function as cell cycle–regulated ubiquitin-protein ligases that mediate destruction box–dependent ubiquitination (*4*, *5*) and thereby target cyclins for proteolysis by the proteasome (*6*). The isolation of mutants defective in cyclin degradation led to the identification of the tetratricopeptide repeat proteins Cdc16p, Cdc23p, and Cdc27p as subunits of the APC (*4*, *7*, *8*). These proteins are required for the onset of anaphase in various organisms (*9*, *10*). Because cyclin proteolysis per se is not required for anaphase (*2*, *11*), it has been suggested that the APC also targets for destruction proteins whose degradation is necessary for sister chromatid separation (*7*, *12*).

Mitotic cyclins such as Clb2p are rapidly degraded in G_1-arrested yeast cells (*7*, *13*). To isolate mutants defective in cyclin proteolysis, we screened mutagenized colonies for β-galactosidase activity resulting from the accumulation of a Clb2-lacZ protein in G_1 at 37°C as described (*7*). We identified 18 mutants that arrested as large, budded cells with a 2C DNA content after cycling cultures were shifted from 25° to 37°C (*14*). Complementation

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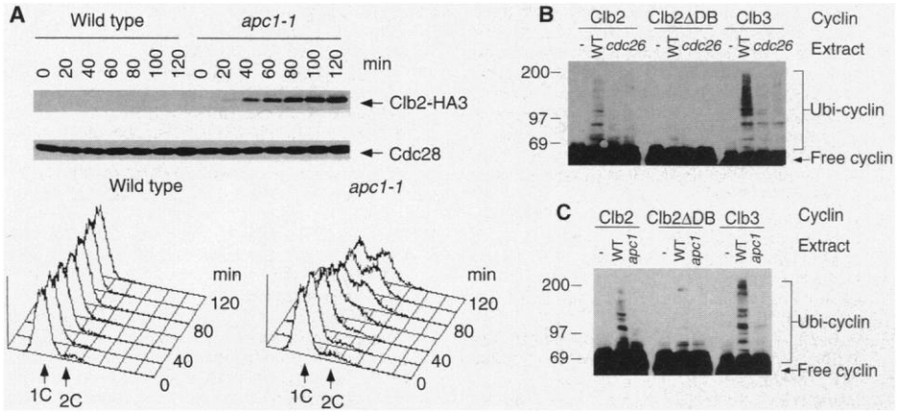

Fig. 1. Defective proteolysis and ubiquitination of mitotic cyclins in the *cdc26-519* and *apc1-1* mutants. (**A**) Accumulation of Clb2p in G_1-arrested *apc1-1* cells. Wild-type (K5137) and *apc1-1* cells (K6221) of the genotype *MATa GALp-CLB2-HA3 bar1* were arrested in G_1 with α-factor in raffinose medium at 23°C. Cells were transferred to raffinose-galactose medium containing α-factor at 35°C. Samples for immunoblotting and analysis of cellular DNA content were withdrawn at the indicated time points. (**B**) Ubiquitin-conjugating activity in extracts from a wild-type (K5518) (WT) and two *cdc26-519* strains (K6200 and YWZ135). Strains (*MATa pep4 bar1*) were arrested in G_1 with α-factor at 25°C and shifted to 37°C for 90 min. Extracts were incubated for 5 min with an adenosine triphosphate–regenerating system and the indicated HA3-tagged cyclin substrate as described (*8*). Clb2ΔDBp lacks the destruction box (*2*). Cyclin-ubiquitin conjugates were detected by immunoblotting with an antibody to HA. (**C**) Cyclin ubiquitination in extracts from G_1-arrested wild-type (K1771) and *apc1-1* cells (K6199). Strains were arrested with α-factor at 23°C and shifted to 37°C for 40 min. Molecular sizes are indicated on the left (in kilodaltons).

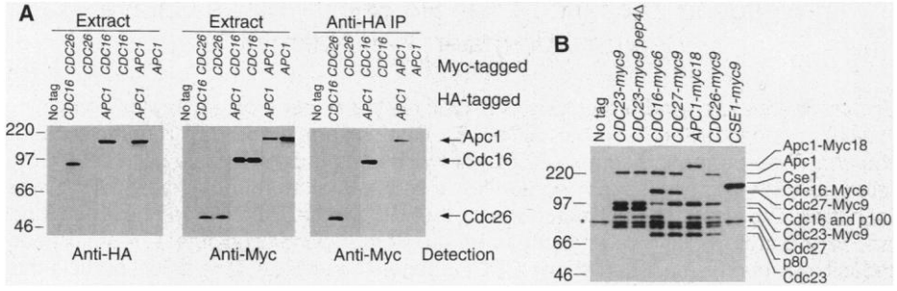

Fig. 2. Coimmmunoprecipitation of Apc1p and Cdc26p with other subunits of the APC. (**A**) Association of Cdc26p and Apc1p with Cdc16p, and self-association of Apc1p. Extracts were prepared from strains expressing Myc- and HA-tagged proteins as indicated and subjected to immunoprecipitations with the antibody to HA (*24*). Epitope-tagged proteins in the extracts and the immunoprecipitates (Anti-HA IP) were detected by immunoblotting with the antibodies 12CA5 (Anti-HA) or 9E10 (Anti-Myc). (**B**) Subunits of the yeast APC. Cells of several strains, each expressing a different Myc-tagged protein, were labeled with ^{35}S-methionine and ^{35}S-cysteine. Extracts were prepared and subjected to immunoprecipitations with the antibody 9E10 (anti-Myc) (*24*). Bound proteins were detected by fluorography. A protein whose precipitation does not depend on the Myc epitope tag is marked with an asterisk.

analysis and gene cloning demonstrated that we had isolated seven mutant alleles of *CSE1*, four of *CDC16*, one of *CDC26* (*cdc26-519*), and a mutant allele of a gene that we called *APC1* (*15*). *cse1* and *cdc16* mutants have been identified previously in a similar screen (*7*). The *CDC26* gene encodes an acidic protein of 17 kD (*16*).

Induction of a *GAL* promoter–*CLB2-HA3* fusion in G_1-arrested cells at 35°C resulted in the accumulation of Clb2-HA3p within 20 min in the *apc1-1* mutant but not in wild-type cells. Mutant cells initiated S phase after 60 min but failed to bud, whereas wild-type cells stayed arrested (Fig. 1A). A similar phenotype is caused by expression of a nondestructible form of Clb2p in wild-type cells (*13*). Extracts prepared from G_1-arrested *cdc26-519* and *apc1-1* mutants were defective in ubiquitination of the mitotic cyclins Clb2p and Clb3p (Fig. 1, B and C), suggesting that reduced Clb2p proteolysis in the mutants stems from defective ubiquitination.

At 37°C, *cdc26-519* mutants arrested as large, budded cells containing a short mitotic spindle in an undivided nucleus positioned at the bud neck. Haploid cells lacking the entire *CDC26*-coding sequence were viable at 25°C but arrested with a similar phenotye at 37°C (*17*). This result confirms that *CDC26* is only essential at increased temperatures (*16*). Epitope tagging revealed that the amount of Cdc26p increased 10-fold when cells grown at 25°C were shifted to 37°C. In contrast, the amount of Cdc16p did not increase (*18*).

The *APC1* wild-type gene (*15*) encodes a 1748–amino acid (192-kD) protein whose COOH-terminal half is similar to the BIME protein from *Aspergillus nidulans* (*19*) and to a related protein encoded by the mouse *tsg24* gene (*20*). An alignment of amino acids 904 to 1713 of Apc1p shows 28 and 25% identity to the corresponding regions of BIMEp (amino acids 1217 to 1939) and Tsg24p (amino acids 1026 to 1777), respectively (*21*). Tetrad analysis of spores derived from a diploid in which one copy of *APC1*

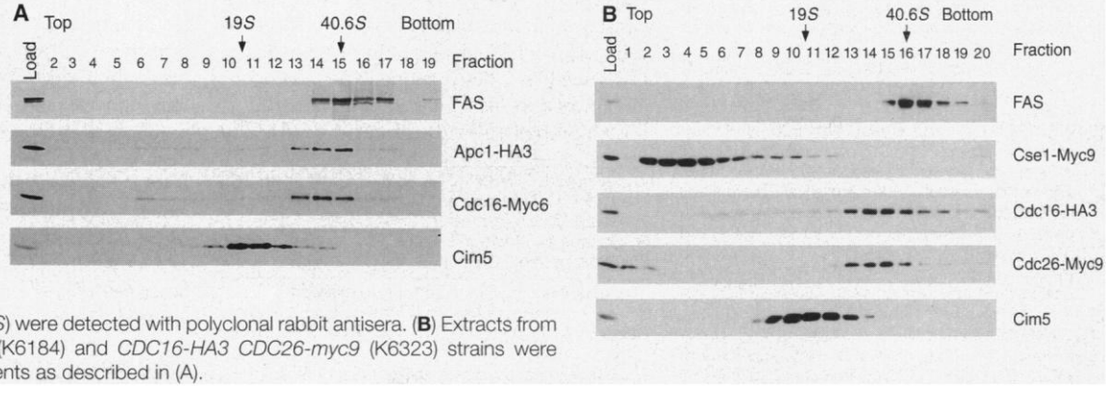

Fig. 3. Cosedimentation of Apc1p, Cdc16p, and Cdc26p as a 36S particle. (**A**) An extract from an *APC1-HA3 CDC16-myc6* strain (K6190) was separated in a 10 to 35% glycerol gradient, and fractions were analyzed by immunoblotting (*25*). Cim5p, a subunit of the 19S proteasome-activator complex, and fatty acid synthetase (FAS, 40.6S) were detected with polyclonal rabbit antisera. (**B**) Extracts from *CDC16-HA3 CSE1-myc9* (K6184) and *CDC16-HA3 CDC26-myc9* (K6323) strains were separated on parallel gradients as described in (A).

was replaced by *HIS3* showed that *APC1* is an essential gene (*22*). His$^+$ spores arrested as large, budded cells after one or two cell divisions after germination.

Cdc16p, Cdc23p, and Cdc27p associate with each other in yeast (*10*). Cyclin proteolysis in vivo and cyclin ubiquitination in vitro also depends on the Cse1 protein (*7*, *8*). To investigate whether Cdc26p, Apc1p, and Cse1p associate with the Cdc16p-Cdc23p-Cdc27p complex, we modified the endogenous genes to encode variants carrying COOH-terminal hemagglutinin (HA) or Myc epitope tags (*23*). All of these variants were fully functional. Extracts were prepared from strains expressing two proteins with different epitope tags and subjected to immunoprecipitations with the antibody to HA (*24*) (Fig. 2A). Cdc16-Myc6p was coprecipitated with Apc1-HA3p but not with Cse1-HA3p. Cdc26-Myc9p was coprecipitated with Cdc16-HA3p. Apc1-Myc6p was coprecipitated with Apc1-HA3p from an extract prepared from an *APC1-HA3/APC1-myc6* diploid strain. To characterize the complex further, we labeled various strains, each expressing a different Myc-tagged protein, with ^{35}S-methionine and ^{35}S-cysteine (*24*) (Fig. 2B). The antibody to the Myc epitope precipitated the same set of proteins from extracts of *CDC16-myc6*, *CDC23-myc9*, *CDC26-myc9*, *CDC27-myc9*, and *APC1-myc18* cells. This set included Cdc16p, Cdc23p, Cdc27p, and Apc1p, which were identified by the increased molecular size of epitope-tagged variants, and a protein of 80 kD. A protein with a size close to that of Cdc16p (100 kD) was detected in the immunoprecipitate from the *CDC16-myc6* strain. None of these proteins was coprecipitated with Cse1-Myc9p. These data, together with previous work (*8*, *10*), suggest that cyclin ubiquitination in yeast depends on a complex containing at least two molecules each of Apc1p, Cdc16p, Cdc23p, and Cdc27p. Cdc26p and at least two unidentified proteins (p80 and p100) are also components of this complex.

To determine the size of the yeast APC, we analyzed extracts from strains expressing two epitope-tagged proteins by glycerol den-

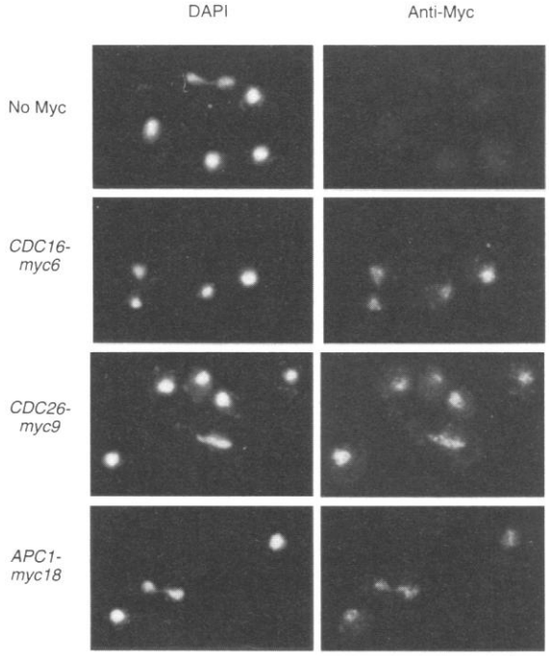

Fig. 4. Nuclear localization of subunits of the yeast APC. Control cells (No Myc, K1534) and cells containing *CDC16-myc6* (K6180), *CDC26-myc9* (K6322), or *APC1-myc18* (K6329) were fixed, and Myc-tagged proteins were detected by indirect immunofluorescence (*26*). DNA was stained with 4′,6′-diamidino-2-phenylindole (DAPI).

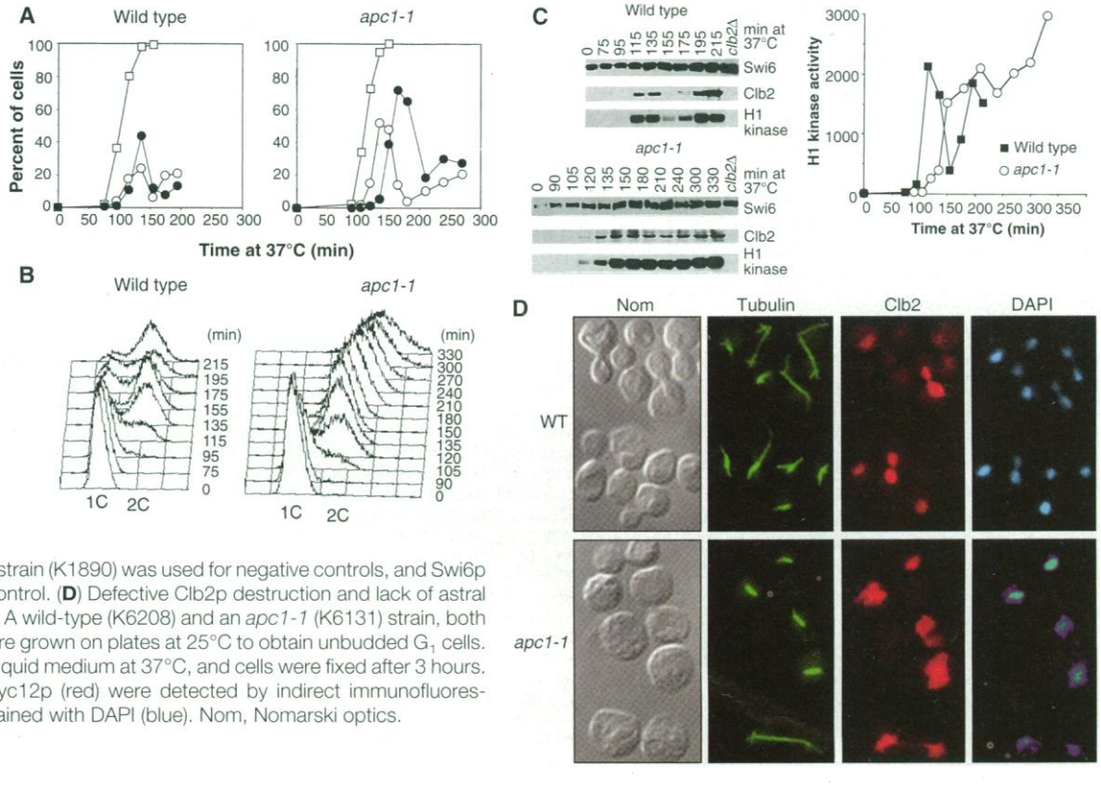

Fig. 5. Defective anaphase in the *apc1-1* mutant. Small, unbudded G_1 cells were isolated by centrifugal elutriation from a wild-type (K699) and an *apc1-1* strain (K5717) grown at 25°C. Cells were released into fresh medium at 37°C, and samples were withdrawn at the indicated time points (*28*). (**A**) Percentage of budded cells (□), of cells containing a short spindle in an undivided nucleus (○), and of cells with separated chromosomes and an elongated spindle (late anaphase–telophase) (●). (**B**) Distribution of the DNA content. (**C**) Clb2 protein and Clb2p-associated kinase activity. A *clb2* deletion strain (K1890) was used for negative controls, and Swi6p was detected as a loading control. (**D**) Defective Clb2p destruction and lack of astral microtubules in *apc1-1* cells. A wild-type (K6208) and an *apc1-1* (K6131) strain, both containing *CLB2-myc12*, were grown on plates at 25°C to obtain unbudded G_1 cells. Strains were inoculated into liquid medium at 37°C, and cells were fixed after 3 hours. Tubulin (green) and Clb2-Myc12p (red) were detected by indirect immunofluorescence (*26*), and DNA was stained with DAPI (blue). Nom, Nomarski optics.

sity gradient centrifugation (*25*). Apc1-HA3p and Cdc23-HA3p cosedimented with Cdc16-Myc6p as a 36S particle [Fig. 3A and (*18*)]. This size is larger than that reported for the *Xenopus* APC (20S) (*4*) and the cyclosome from clam (*5*). Cdc26-Myc9p but not Cse1-Myc9p cosedimented with Cdc16-HA3p (Fig 3B). These results and the immunoprecipitation data indicate that Cse1p is not a component of the APC.

The subcellular localization of the APC components was determined by indirect immunofluorescence (*26*). Cdc16-Myc6p, Cdc23-Myc9p, Cdc26-Myc9p, Cdc27-Myc9p, and Apc1-Myc18p are all localized in the nucleus [Fig. 4 and (*18*)]. Cse1-Myc9p has also been shown to be a nuclear protein (*27*). We did not detect specific accumulation of any of these proteins at spindle pole bodies or on mitotic spindles.

To investigate the role of *APC1* in vivo, we isolated small, unbudded G_1 cells from wild-type and *apc1-1* cultures grown at 25°C and followed their progression through the cell cycle upon incubation at 37°C (*28*). In *apc1-1* cells, DNA replication, budding, and the formation of mitotic spindles occurred at the same time as in wild-type cells, but entry into anaphase was delayed by about 20 min (Fig. 5, A and B). Most *apc1-1* cells later underwent anaphase but were slow in disassembling their mitotic spindles. They eventually rebudded without undergoing cytokinesis or re-replication. We conclude that *apc1-1* cells are defective in the onset of anaphase, in the final exit from mitosis, and in the completion of cytokinesis. In the *apc1-1* mutant, Clb2 protein and Clb2p-associated histone H1 kinase activity appeared later than in wild-type cells. This finding could explain why entry into anaphase is delayed in the mutant. Neither Clb2 protein nor Clb2p-Cdc28p kinase activity declined as mutant cells underwent anaphase (Fig. 5C). The observation that *apc1-1* cells rebud without any apparent decrease in kinase activity is surprising because high Clbp-Cdc28p kinase activity is thought to inhibit rebudding (*3*).

To detect Clb2p in individual cells by indirect immunofluorescence microscopy (*26*), we replaced the endogenous *CLB2* genes of a wild-type and an *apc1-1* strain by the epitope-tagged variant *CLB2-myc12* (*23*). G_1 cells, obtained by growth to stationary phase at 25°C, were inoculated into fresh medium at 37°C, causing cells to reenter the cell cycle. In wild-type cells, Clb2-Myc12p accumulated in maximal amounts at the onset of anaphase and then declined rapidly as cells underwent nuclear division. In the *apc1-1* mutant, the amount of Clb2-Myc12p remained high in cells containing separated chromosomes and fully elongated spindles (Fig. 5D). We conclude that Apc1p is required for cyclin proteolysis not only in G_1 but also in late anaphase–telophase. Surprisingly, *apc1-1* cells were defective in the formation of astral microtubules emanating from the poles of mitotic spindles (Fig. 5D). In contrast, *cdc16-123* cells arrested at 37°C had normal astral microtubules (*18*).

The accumulation of Cdc26p at high temperature is consistent with the observation that Cdc26p function is only essential for APC activity at 37°C. Cdc26p may be required to stabilize the APC or to modulate its activity under conditions of stress, such as heat shock. The finding that the BIMEp homologs of yeast (Apc1p) and frog (*29*) are subunits of the APC explains the pre-anaphase arrest of *bimE* mutants. Loss of *bimE* function partially bypasses the control mechanisms that render entry into mitosis dependent on the completion of DNA replication and on the activation of the NIMA kinase (*30*). Taken together, these data indicate that the APC is not only required for the onset of anaphase and the exit from mitosis but may also participate in regulating entry into mitosis.

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14. Strain K3828 was mutagenized and colonies (400,000) of G_1-arrested cells were screened for accumulation of Clb2-lacZp at 37°C (*7*). Mutants were further analyzed that arrested as large, budded cells with a 2C DNA content after cycling cultures were shifted from 25° to 37°C. The original mutants were backcrossed at least three times to wild type.
15. Mutants were transformed with a genomic library [F. Cvrckova and K. Nasmyth, *EMBO J.* **12**, 5277 (1993)], and plasmids allowing growth at 37°C were recovered. The *apc1-1* mutation was complemented by the open reading frame YNL172W on chromosome XIV (*Saccharomyces* Genome Database). The *cdc26-519* and *apc1-1* mutants were crossed to strains containing a *CDC26::LEU2* or an *APC1::HIS3* fusion, respectively. All tetrads were parental ditypes.
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17. One *CDC26* allele of a diploid strain was replaced by the *Kluyveromyces lactis URA3* gene. Tetrads consisted of two Ura^+ spores that were viable at 25°C but arrested as large, budded cells at 37°C and two Ura^- spores that grew normally at 37°C.
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21. T. H. Shin and K. Nasmyth, unpublished results.
22. A 4.3-kb Bam HI to Sty I fragment was replaced by *HIS3*, and the *apc1::HIS3* construct was used to disrupt one *APC1* allele in a diploid strain.
23. Two hundred base pairs (bp) of coding region with several HA or Myc epitope tags inserted in front of the stop codon and 200 bp of 3′-noncoding sequence were cloned into an integrative vector. The resulting plasmid was integrated at the respective genomic locus. Strains expressing two epitope-tagged proteins were obtained by genetic crosses. At 37°C all strains, including a *clb1Δ CLB2-myc12* strain, grew normally, demonstrating that the epitope-tagged proteins were fully functional.
24. Extracts for immunoprecipitations were prepared as described (*10*). Cells (2×10^9) grown in YEP medium (1% yeast extract, 1% peptone) with 2% glucose were broken in 0.4 ml of solution A containing 10% glycerol and 1 mM dithiothreitol. Extracts (6 mg, 0.3 ml) were incubated with the 12CA5 antibody cross-linked to protein A–Sepharose (30 μl), and bound proteins were analyzed by immunoblotting. For metabolic labeling, strains were grown in SC medium lacking methionine [F. Sherman, *Methods Enzymol.* **194**, 3 (1991)]. Cells (5×10^7) were labeled with 1 mCi of ^{35}S-methionine and ^{35}S-cysteine in 1.5 ml of medium for 2 hours at 30°C. Cells were broken in 0.25 ml of buffer B [buffer A (*8*) diluted 1:3] containing 1.5 mg of unlabeled protein from a *pep4* strain (K5517) (*8*). After centrifugation (10 min, 12,000*g*), extracts were sequentially incubated with protein A–Sepharose (160 μl) and antibody 9E10 cross-linked to protein A–Sepharose (25 μl). The beads were washed with buffer B (4×1 ml), buffer B with 120 mM K acetate (1 ml), and buffer B with 150 mM K acetate (1 ml). Bound proteins were separated on SDS-polyacrylamide gels and detected by fluorography.
25. Cells were grown in YEP medium with 2% raffinose. Extracts (2 mg of protein) prepared in buffer C [buffer A (*8*) without glycerol, diluted 1:3] were layered on 10 to 35% glycerol gradients in buffer C and centrifuged for 15 hours at 28,000 rpm in a Beckman SW40 rotor.
26. Cells were prepared for indirect immunofluorescence [K. Nasmyth, G. Adolf, D. Lydall, A. Seddon, *Cell* **62**, 631 (1990)], and pictures were taken on Kodak T-MAX400 film or with a charge-coupled device camera mounted on a Zeiss Axiophot microscope. Myc-tagged proteins were detected with the 9E10 antibody and a CY3-conjugated secondary antibody. Spindles were detected with a rabbit antiserum to yeast tubulin and a fluorescein isothiocyanate–conjugated secondary antibody.
27. R. Ciosk and K. Nasmyth, unpublished results.
28. Strains were grown in SC medium [F. Sherman, *Methods Enzymol.* **194**, 3 (1991)] containing 2% raffinose at 25°C. Small, unbudded cells were isolated by centrifugal elutriation [E. Schwob and K. Nasmyth, *Genes Dev.* **7**, 1160 (1993)] and inoculated into YEP medium with 2% glucose at 37°C. Clb2-associated histone H1 kinase (*2*) was quantified with a PhosphorImager (Molecular Dynamics). A FACScan (Becton-Dickinson) was used for analysis of cellular DNA content [C. B. Epstein and F. R. Cross, *Genes Dev.* **6**, 1695 (1992)].
29. J. M. Peters, R. W. King, C. Höög, M. W. Kirschner, *Science* **274**, 1199 (1996).
30. S. A. Osmani, D. B. Engle, J: H. Doonan, N. R. Morris, *Cell* **52**, 241 (1988); S. W. James, P. M. Mirabito, P. C. Scacheri, N. R. Morris, *J. Cell Sci.* **108**, 3485 (1995).

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