re-replication were completely blocked [Fig. 5, B and C; the wild-type strain was analyzed in (*27*)]. Degradation of Pds1p, which starts shortly before anaphase, is required for sister chromatid separation (*5*, *27*). Detection of Pds1-Myc18p revealed that arrested *apc2-1* cells contained large amounts of Pds1p (Fig. 5C). Deletion of the *PDS1* gene allowed *apc2-1* cells to separate sister chromatids (Fig. 5D). However, spindle elongation was slower in *apc2-1* Δ*pds1* cells than in wild-type cells. Thus, the inability of *apc2-1* cells to enter anaphase may result primarily from a defect in the degradation of Pds1p. *apc2-1* cells were also defective in degrading the mitotic cyclin Clb2p (*18*). Extracts prepared from G_1-arrested *apc2-1* and *apc2-2* cells were defective in the ubiquitination of mitotic cyclins (Fig. 5E), indicating that the defect in proteolysis results from defective ubiquitination.

Yeast Apc5p shows similarity to human Apc5p (*21*) and to the putative ORF M163.4 from *C. elegans*. The yeast Apc4p sequence shows weak similarity to the human Apc4p sequence (*21*) and to the ORF Z97209 from *Schizosaccharomyces pombe*, which is more closely related to the human protein. Apc4p might represent an APC component that has diverged more during evolution than the other subunits. No homologs have been identified for Apc9p. Thus, in addition to Apc1p, Cdc16p, Cdc23p, Cdc27p, and Apc10p/Doc1p, at least Apc2p, Apc5p, and Apc11p might be conserved subunits of the APC in all eukaryotes.

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11. ^{35}S-Labeled cells (*8*) were broken in 0.25 ml of buffer B [50 mM Hepes-KOH (pH 7.3), 5 mM Mg acetate, 0.1% Triton X-100, 20 mM β-glycerophosphate, 10% glycerol, 1 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride pepstatin (1 μg/ml), proteinase inhibitors (Complete, Boehringer)] containing 60 mM K acetate and bovine serum albumin (BSA, 5 mg/ml). After centrifugation (10 min, 18,000*g*), extracts were incubated with protein A–Sepharose (0.17 ml) for 30 min and with antibody 9E10 cross-linked to protein A–Sepharose (9E10-beads, 27 μl) for 90 min. Beads were washed three times with 1 ml of buffer B100 (numbers indicate the K acetate concentration in millimolar) containing BSA and with 1 ml of buffers B100, B120, and B150, containing insulin (0.1 mg/ml). Prestained proteins (Rainbow, Amersham) were used as molecular size markers.
12. To identify p90 and p70, *CDC16-myc6* or *CDC23-myc9* cells (10^{10}) (Δ*pep4*) were broken in buffer B70 [4 ml (*11*)]. Extracts were centrifuged twice (20 min, 18,000*g*) and incubated with protein A–Sepharose (2 ml) for 1 hour and with 9E10-beads (0.2 ml) for 2.5 hours. Beads were washed three times with 4 ml of buffer B100, B150, and B200 containing insulin (0.1 mg/ml) and with 2 ml of buffer B containing 50 mM Na acetate. Proteins eluted with SDS were separated on SDS-polyacrylamide gels. p40 and p23 were identified in immunoprecipitates from 5.5×10^{10} cells.
13. Proteins were visualized by silver staining and digested in the gel with trypsin [A. Shevchenko, M. Wilm, O. Vorm, M. Mann, *Anal. Chem.* **68**, 850 (1996)]. The digest was analyzed by nanoelectrospray tandem mass spectrometry [M. Wilm *et al.*, *Nature* **379**, 466 (1996)]. Parent ion scans for the immonium ions of leucine and isoleucine were used to detect peptide ions [M. Wilm, G. Neubauer, M. Mann, *Anal. Chem.* **68**, 527 (1996)]. Relative to a BSA standard, the amount of protein available for mass spectrometric identification was ~10 to 20 ng per band.
14. ORF designations are from the *Saccharomyces* genome database.
15. A. Shevchenko and M. Mann, unpublished results.
16. All strains are W303 derivatives. ORFs were tagged at the COOH-terminus with three hemagglutinin A (HA) or nine Myc epitopes (*8*). At 37°C, *DOC1-HA3*, *DOC1-myc9*, and *APC11-myc9* strains arrested in mitosis. All other strains (including *APC11-HA3*) grew normally, indicating that these tagged proteins were fully functional. ORFs were replaced with a *S. pombe his5*$^+$ cassette amplified from pFA6a-HIS3MX6 [A. Wach, A. Brachat, C. Alberti-Segui, C. Rebischung, P. Philippsen, *Yeast* **13**, 1065 (1997)].
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26. *apc2* mutants were generated as described [S. H. MacKelvie, P. D. Andrews, M. J. R. Stark, *Mol. Cell. Biol.* **15**, 3777 (1995)]. The Xho I to Nde I fragment was removed from the *APC2* gene (Cla I to Bgl II) in a CEN-*LEU2* plasmid. This DNA was transformed, together with a mutagenized *apc2* fragment (−29 to +2554, ATG = +1), into a Δ*apc2::TRP1* strain containing a CEN-*URA3-APC2* plasmid. After selection of transformants showing cell cycle arrest at 37°C, mutant *apc2* alleles and the wild-type gene were integrated at the *his3* locus of the Δ*apc2::TRP1* strain.
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28. Small G_1 cells were elutriated from strains grown at 21°C [E. Schwob and K. Nasmyth, *Genes Dev.* **7**, 1160 (1993)]. Flow cytometric DNA quantitation, indirect immunofluorescence (to detect Pds1-Myc18p and spindles), and observation of tetR-GFP were done as described (*27*).
29. Amino acids: 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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Identification of a Cullin Homology Region in a Subunit of the Anaphase-Promoting Complex

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The anaphase-promoting complex is composed of eight protein subunits, including BimE (APC1), CDC27 (APC3), CDC16 (APC6), and CDC23 (APC8). The remaining four human APC subunits, APC2, APC4, APC5, and APC7, as well as human CDC23, were cloned. APC7 contains multiple copies of the tetratrico peptide repeat, similar to CDC16, CDC23, and CDC27. Whereas APC4 and APC5 share no similarity to proteins of known function, APC2 contains a region that is similar to a sequence in cullins, a family of proteins implicated in the ubiquitination of G_1 phase cyclins and cyclin-dependent kinase inhibitors. The *APC2* gene is essential in *Saccharomyces cerevisiae*, and *apc2* mutants arrest at metaphase and are defective in the degradation of Pds1p. APC2 and cullins may be distantly related members of a ubiquitin ligase family that targets cell cycle regulators for degradation.

Two distinct ubiquitin-mediated proteolytic pathways regulate the G_1 to S phase and metaphase to anaphase transitions during the cell division cycle (*1*). In late G_1, a CDC34-dependent ubiquitination pathway degrades Sic1p, an inhibitor of G_1 cyclin-dependent kinases in budding yeast, enabling the onset of S phase (*2*); a similar pathway exists in metazoans (*3*). A distinct ubiquitination machinery governs exit from

mitosis through the degradation of mitotic cyclins and other regulatory proteins, such as Pds1p and Ase1p in budding yeast and Cut2 in fission yeast (*4*–*8*). In this system, a large protein complex, termed the anaphase-promoting complex (APC) or the cyclosome, functions as a protein ubiquitin ligase (*9*–*12*). In the presence of ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes such as UBCx (also called E2-C in clam and UBC-H10 in human) or UBC4, the APC catalyzes the formation of cyclin-ubiquitin conjugates (*9*, *13*–*15*), which are subsequently degraded by the 26S proteasome (*16*). The APC is the cell cycle–regulated component of the mitotic cyclin degradation system (*9*, *17*, *18*). The APC is also thought to participate in substrate recognition, which depends on a sequence element found in all APC substrates, called the destruction box (*4*, *13*, *19*). Biochemical studies have shown that the vertebrate APC contains eight subunits, named APC1 to APC8. These subunits include BimE (APC1), CDC27 (APC3), CDC16 (APC6), and CDC23 (APC8) (*9*, *11*, *17*).

We report the cDNA cloning of the remaining four human APC subunits on the basis of peptide sequence information obtained from immunopurified *Xenopus* APC (*17*, *20*). We first identified human expressed sequence tags (ESTs) that encode amino acid sequences more than 80% identical to the *Xenopus* APC peptides (*20*). On the basis of the sequences of the ESTs, we cloned the full-length human cDNAs for APC2, APC4, APC5, and APC7 (*21*) (Fig. 1A). The human homolog of *Saccharomyces cerevisiae CDC23* (APC8) was also isolated (*21*). A CDC23 peptide was isolated from the APC7 band, indicating that APC7 was CDC23 (*17*). Subsequent sequencing of more peptides from APC7 and APC8 bands and the analysis of the APC7 and APC8 cDNA

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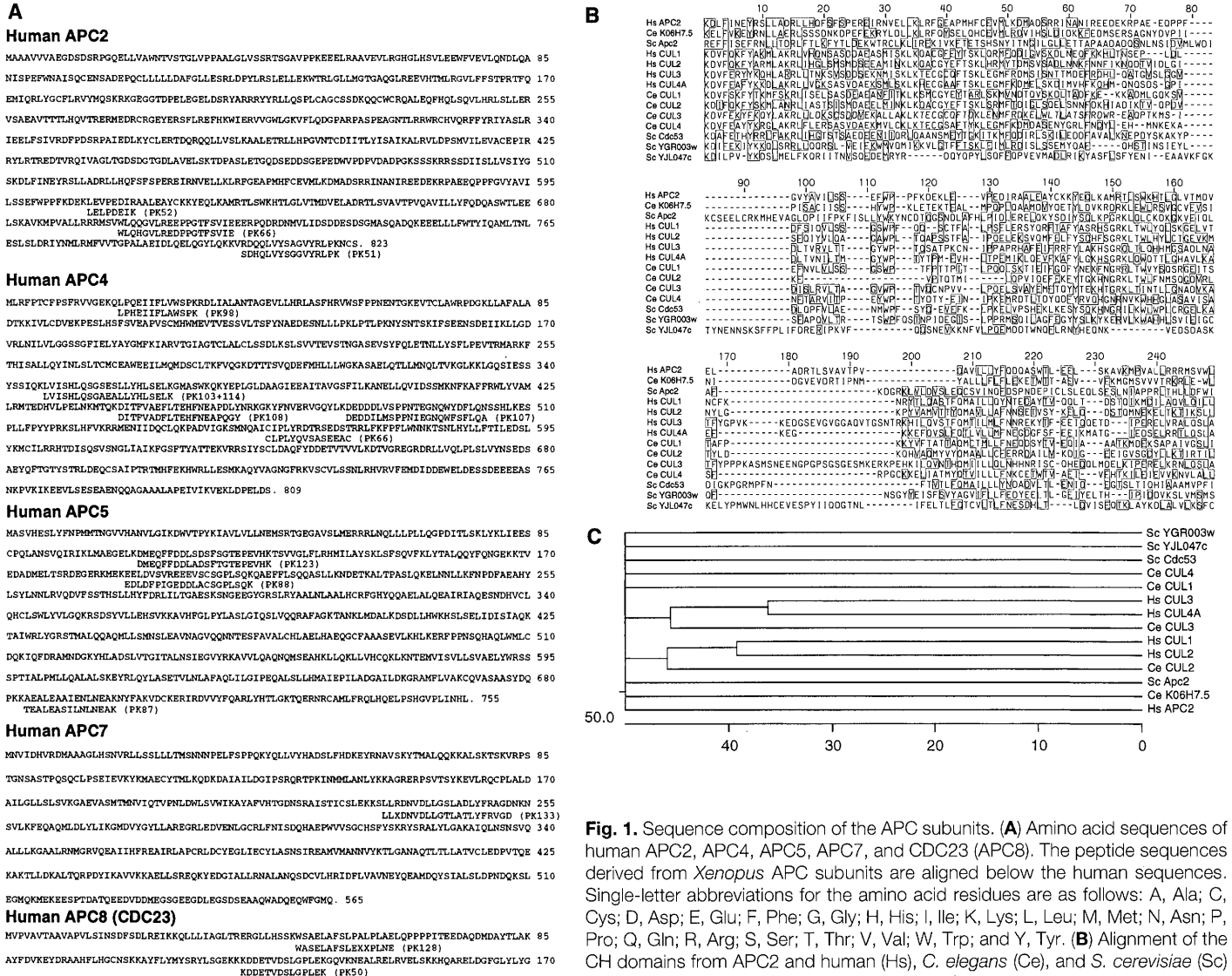

Fig. 1. Sequence composition of the APC subunits. (**A**) Amino acid sequences of human APC2, APC4, APC5, APC7, and CDC23 (APC8). The peptide sequences derived from *Xenopus* APC subunits are aligned below the human sequences. Single-letter abbreviations for the amino acid residues are as follows: A, 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. (**B**) Alignment of the CH domains from APC2 and human (Hs), *C. elegans* (Ce), and *S. cerevisiae* (Sc) cullins. The alignment was made with the Clustal method with the program MEGALIGN (DNASTAR, Madison, WI). Residues identical to the consensus are boxed in black. (**C**) Phylogenetic tree of CH domains.

clones by in vitro translation and immunoblotting showed that APC8 is the CDC23 homolog, whereas APC7 is a new APC subunit. We suspect that the CDC23 peptide was isolated from the APC7 band because of insufficient separation of these subunits by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).

Database searches revealed that APC4 and APC5 do not share sequence similarity with proteins of known function. APC4 does not appear to have a homolog in *S. cerevisiae*. However, the NH_2-terminal 450 residues of APC4 share limited similarity with the *Schizosaccharomyces pombe* open reading frame (ORF) Z97209, which in turn is distantly related to the *S. cerevisiae* ORF YDR118w. The YDR118w protein is a subunit of the yeast APC (APC4) (*22*). APC5 is similar to a putative *S. cerevisiae* protein, ORF YOR249c. Deletion of this ORF from the *S. cerevisiae* genome indicates that YOR249c is essential for viability, and the null mutants exhibit a terminal G_2-M arrest phenotype as would be expected for APC genes (*23*). The YOR249c protein is found in the purified *S. cerevisiae* APC (*22*). Like CDC16, CDC23, and CDC27, APC7 is a tetratrico peptide repeat–containing protein that is most similar to Cdc27p in the yeast genome.

Database searching with the APC2 sequence revealed that it is similar to a recently identified family of proteins, called cullins (*24*). The similarity between APC2 and cullins is restricted to a 200–amino acid region, which we refer to as the cullin homology (CH) region (Fig. 1, B and C). A cullin protein, Cdc53p, is part of a ubiquitin ligase complex that targets phosphorylated Sic1p and G_1 cyclins for degradation in budding yeast (*25*). Mutations in the *Caenorhabditis elegans cul-1* gene cause hyperplasia of all tissues, which would be consistent with a defect in G_1 cyclin degradation (*24*). Several human cullins have also been identified in the EST database (*24*). Therefore, cullins represent a conserved family of proteins that may be part of the ubiquitin ligases for the degradation of Sic1p, G_1 cyclins, and other regulatory proteins (*24*, *25*). Two tandem *C. elegans* ORFs K06H7.6 and K06H7.5 on cosmid K06H7 are similar to the NH_2- and COOH-terminal regions of human APC2, respectively, and probably represent a single gene. Furthermore, we identified a hypothetical yeast protein, ORF YLR127c, which is 18% identical (34% similar) to human APC2 ($P = 1.5 \times 10^{-20}$). To determine whether YLR127c encodes an APC subunit in budding yeast, we cloned the gene encoding YLR127c and inserted a triple hemagglutinin (HA) epitope tag at the NH_2-terminus (*26*). The epitope-tagged protein efficiently rescued an *apc2* deletion mutation (*apc2::HIS3*). HA-tagged yeast Apc2p coimmunoprecipitated with three subunits of the budding yeast APC: Cdc27p, Cdc16p, and Cdc23p (Fig. 2). Apc2p did not coimmunoprecipitate with Cdc34p or Cdc53p, however, suggesting that Apc2p is not a shared component between the APC and the Cdc4p-Cdc34p-Cdc53p complex. Cdc53p is much more closely related to ORFs YGR003w and YJL047c than to Apc2p. A database search with the sequence of Cdc53p identified

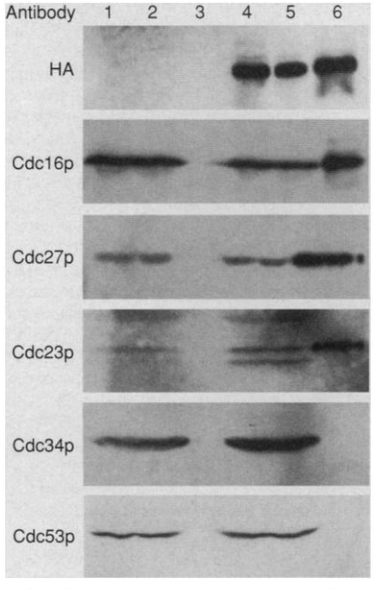

Fig. 2. Coimmunoprecipitation of Cdc16p, Cdc27p, and Cdc23p, but not Cdc34p or Cdc53p, with Apc2-3XHA from budding yeast extracts. Extracts were prepared from logarithmic phase cells of the yeast strain YAP89 expressing Apc2p-3XHA from its own promoter (lanes 4 to 6) or from a congenic strain expressing untagged Apc2p, YAP87 (lanes 1 to 3), and immunoprecipitated with antibody to HA sepharose beads. Crude cell extract (lanes 1 and 4), supernatants (lanes 2 and 5), and the immunoprecipitates (lanes 3 and 6) were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with the indicated antibodies.

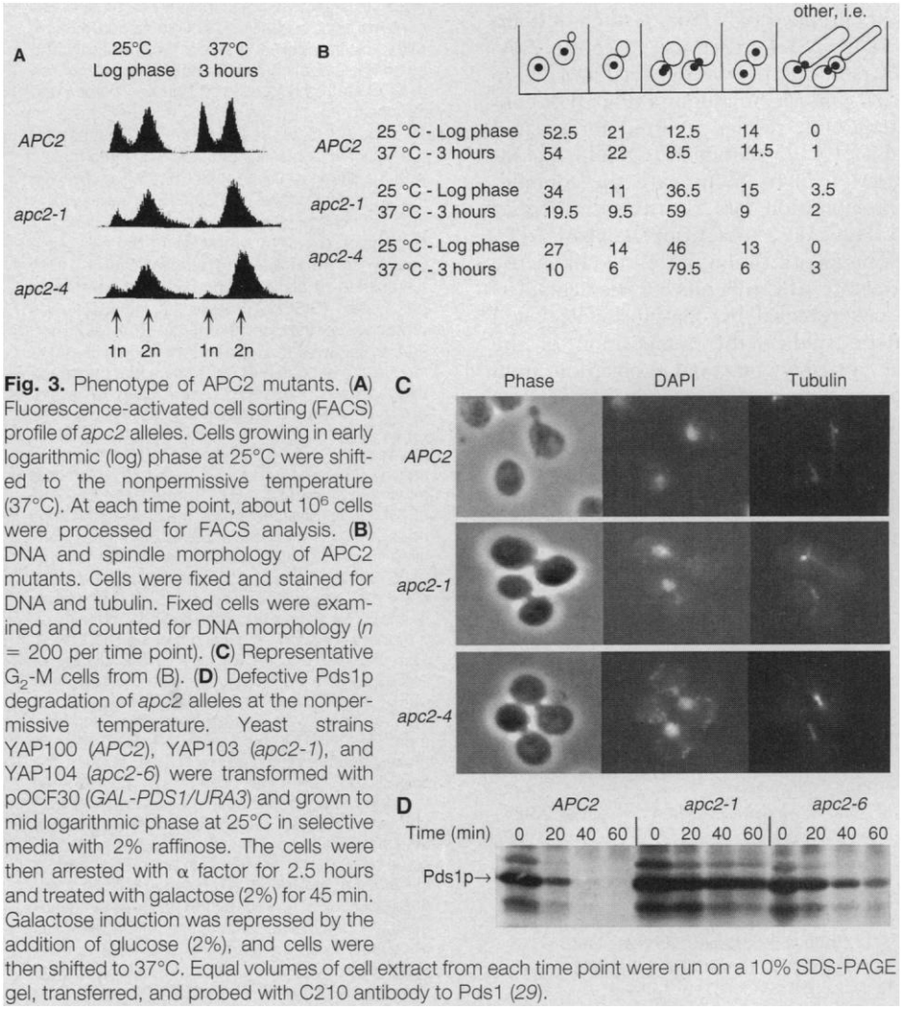

Fig. 3. Phenotype of APC2 mutants. (**A**) Fluorescence-activated cell sorting (FACS) profile of *apc2* alleles. Cells growing in early logarithmic (log) phase at 25°C were shifted to the nonpermissive temperature (37°C). At each time point, about 10^6 cells were processed for FACS analysis. (**B**) DNA and spindle morphology of APC2 mutants. Cells were fixed and stained for DNA and tubulin. Fixed cells were examined and counted for DNA morphology (n = 200 per time point). (**C**) Representative G_2-M cells from (B). (**D**) Defective Pds1p degradation of *apc2* alleles at the nonpermissive temperature. Yeast strains YAP100 (*APC2*), YAP103 (*apc2-1*), and YAP104 (*apc2-6*) were transformed with pOCF30 (*GAL-PDS1/URA3*) and grown to mid logarithmic phase at 25°C in selective media with 2% raffinose. The cells were then arrested with α factor for 2.5 hours and treated with galactose (2%) for 45 min. Galactose induction was repressed by the addition of glucose (2%), and cells were then shifted to 37°C. Equal volumes of cell extract from each time point were run on a 10% SDS-PAGE gel, transferred, and probed with C210 antibody to Pds1 (*29*).

YGR003w ($P = 2 \times 10^{-23}$) and YJL047c ($P = 4 \times 10^{-8}$), but the same search could not find APC2p.

We tested whether *APC2* is an essential gene by replacing the complete coding region of YLR127c with the *HIS3* gene (*27*). Tetrad analysis revealed that *APC2* is essential. Dead spores arrested as large or multiply budded cells after one to three cell divisions. We then constructed temperature-sensitive alleles of *APC2* by mutagenesis in the polymerase chain reaction (PCR) and integrated them into the genome at the *LEU2* locus (*28*). Three hours after a shift to the nonpermissive temperature (37°C), two temperature-sensitive alleles, *apc2-1* and *apc2-4*, caused a substantial increase in cells with a 2n DNA content as compared with wild-type cells (Fig. 3, A and B). Both alleles caused cell cycle arrest primarily as large budded cells with the nucleus at or near the neck, a phenotype characteristic of other known APC mutants. Tubulin staining revealed that the majority of cells arrested with short to midlength spindles, indicating a G_2-M arrest (Fig. 3C). The shift to the nonpermissive temperature was lethal; viability dropped to below 7% after 24 hours at 37°C (*23*). Furthermore, a known APC substrate, Pds1p, was stabilized at 37°C in *apc2-1* and *apc2-6* mutants (Fig. 3D), consistent with results reported for *cdc23-1* and *cdc16-123* mutants (*10*). The stabilization of Pds1p, along with the coimmunoprecipitation data, confirms that Apc2p (YLR127c) is a subunit of the yeast APC.

The identification of the stoichiometric vertebrate APC subunits has been completed. As revealed by our biochemical and genetic studies, the composition of the APC is highly conserved in organisms from yeast to humans. Furthermore, APC2 contains a region that shares sequence similarity with cullins. The CH region may perform similar biochemical functions in both systems, such as binding ubiquitin or the ubiquitin-conjugating enzymes.

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20. The *Xenopus* egg extracts and high-speed supernatants (S100) were prepared as previously described (*9*). The interphase S100 was fractionated on a 50-ml Resource Q column (Pharmacia). Antibodies to CDC27 covalently coupled to protein A beads were added to the APC-containing fractions. The beads were washed with 40 volumes of buffer containing 20 mM tris-HCl (pH 7.7), 500 mM KCl, 1 mM $MgCl_2$, and 0.5% NP-40. The APC was then eluted with 100 mM glycine (pH 2.0), separated on SDS-PAGE, transferred to polyvinyl difluoride membrane (Bio-Rad), and processed for microsequencing. We also analyzed the composition and activity of human APC and compared it with that of the *Xenopus* complex. Immunoprecipitation with antibodies to CDC27 from HeLa cell extracts revealed that the human APC also contains eight subunits. APC immunopurified from mitotic HeLa cell extracts was active in ubiquitinating cyclin B1, indicating that the structure and function of APC are highly conserved between human and *Xenopus*.
21. To clone APC5, we searched the EST database at the National Center for Biotechnology Information with the sequence of peptide PK123 (Fig. 1A) with BLAST and found that EST 309267 encodes amino acid sequences 90% identical to PK123. Primers that match the DNA sequence of the EST were used to isolate a full-length clone (APC5-H19) from a human testis plasmid library with the Gene Trapper system (GIBCO BRL) by the manufacturer's protocols. For the cloning of APC7, EST 40875 and EST 41898 were found to encode sequences 81% identical to peptide PK133. Primers that match the sequences of these ESTs were used to isolate APC7-H1 from the human testis library. Because no full-length clones were obtained, the 5′ 300–base pair (bp) region of clone APC7-H1 was amplified by PCR and used as a probe to isolate the full-length clone APC7-H18 from a HeLa Uni-ZAP cDNA library (Stratagene). On the basis of the *Xenopus* peptide sequences, multiple ESTs were identified for CDC23. EST 452573 contained the longest 5′ sequence and was ordered from the American Type Culture Collection. The 5′ 300-bp region of this EST was then amplified by PCR and used as a probe to isolate the full-length clone CDC23-H1 from the HeLa cDNA library. For the cloning of APC2, primers were designed from the sequence of EST 136462 that was identified by the peptides. These primers were used to clone a 1.3-kb fragment (APC2-H1) of the human APC2 cDNA from the testis library with the Gene Trapper system. The 5′ end of APC2-H1 was used as a probe to screen the HeLa library, resulting in the isolation of a 2.1-kb clone, APC2-H7. The 5′ end of APC2-H7 was then amplified and used to isolate the full-length clone APC2-H19 from a human breast cancer cDNA library (MDA468). To clone APC4, we identified a human EST (98516) that encodes sequences 87% identical to one of the APC4 peptides (PK108). Primers corresponding to the 5′ and 3′ regions of this EST were used to amplify a 300-bp DNA fragment from HeLa cDNA (Clontech, Palo Alto, CA) by PCR. This DNA fragment was then used to isolate the full-length clone APC4-H2 from the MDA468 library.
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26. A 4.5-kb Cla I to Eco RI fragment containing YLR127c was subcloned from cosmid 9233 into pRS316 to generate pAP1, which was sufficient to restore the viability of *apc2::HIS3* spores. A cassette containing the *URA3* gene flanked by triple HA repeats and sequences identical to the desired site of integration was amplified from pMPY-3XHA by PCR and transformed into a wild-type haploid strain carrying pAP3 (*TRP1/CEN/APC2*). Plasmid DNA was recovered from URA3 revertants, retransformed into *Escherichia coli*, and digested. Plasmids containing the *URA3* insertion were retransformed into YPH499, and Ura3 revertants were selected on medium containing 5-fluoroorotic acid (5FOA). Extracts from cultured revertants were screened by immunoblotting with the 12CA5 antibody to HA for the presence of a specific band with the expected size.
27. The *HIS3* gene was amplified with a primer pair containing a sequence flanking the YLR127c ORF. The PCR product was transformed into a diploid wild-type strain (YPH501) and selected for on synthetic complete (SC)–His plates. For His-positive transformants, replacement of YLR127c was confirmed by Southern (DNA) blotting with two independent probes.
28. A 3-kb cassette containing the entire *APC2* ORF flanked by about 300 bp of noncoding sequence including the promoter was amplified by PCR and cloned into pRS314. Clones that rescued YAP11, an *APC2* plasmid shuffle strain (an *apc2::HIS3* mutant, rescued by pAP1, a *CEN/URA3/APC2* plasmid), were used as templates for PCR mutagenesis. We used primers that hybridized to pRS vector sequence flanking the multicloning site to amplify a 3.7-kb cassette and cotransformed it with Bam HI–linearized pRS315 into YAP11 or YAP13. After the wild-type copy of *APC2* was shuffled out by growth on 5FOA, transformants were replica-plated twice to SC-Leu and grown at either 25° or 37°. Plasmid DNA was recovered from transformants that successfully retested for temperature sensitivity. A cassette containing the mutant *apc2* allele under its own promoter was subcloned into a *LEU2* integrating vector and transformed into YAP11 or YAP13. Leu-positive transformants were assayed for their ability to lose the wild-type *APC2 URA3* plasmid on 5FOA. The 5FOA-resistant colonies were then screened for their ability to recapitulate the temperature sensitivity and for complementation by episomal wild-type *APC2*.
29. A. Yamamoto, V. Guacci, D. Koshland, *J. Cell Biol.* **133**, 85 (1996).

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