back was altered during the experiment.

11. A sound-pressure level of 60 dB was sufficient to block subjects' ability to hear their own whispering.
12. Informed consent was obtained from all subjects after the nature and possible consequences of the study were explained.
13. Feedback transformations were defined geometrically with respect to a subject's [i]-[a] path. The subject's unaltered formant frequencies were represented as a point in formant space. This point was then rerepresented in terms of two measures: (i) path deviation—the distance to the nearest point on the [i]-[a] path, and (ii) path projection—the position on the [i]-[a] path of this nearest point. The feedback transformation then shifted only the point's path projection; the point's path deviation was preserved.
14. Mean compensation measures how much a subject's mean training word vowel formant change (test phase – baseline) countered the shift of the feedback transformation. It was measured as: (path projection of mean vowel formant change)/(–path projection shift of transform) [see (*13*) for explanation of path projection]. This ratio is 1.0 for perfect compensation. Mean adaptation measured how much compensation was retained in the absence of feedback. Thus, mean adaptation was calculated with the same ratio shown above, except it used only formant data collected when the subject whispered with feedback blocked by noise. (In the control experiment, because feedback was not altered, mean compensation and adaptation for each subject were calculated with respect to the feedback alteration used in the adaptation experiment.)
15. Analysis-of-variance tests of path projection changes (test phase – baseline) across subjects in the adaptation and control experiments were computed from formant data collected when subjects whispered while hearing feedback (for the compensation analysis) or while hearing was blocked by masking noise (for the adaptation analysis). The interaction of experiment type (adaptation versus control) and path projection changes was used to judge significance.
16. For a given test word, mean generalization was computed as: (mean test word relative adaptation)/(mean training word relative adaptation), where relative adaptation was computed by subtracting adaptation seen in the control experiment from that seen in the adaptation experiment.
17. Tests of significant generalization were based on computing the significance of test word adaptations, which were computed the same way as the training word adaptation significance tests described in (*15*).
18. We had technical problems estimating the formants of whispered [i] and [a]; thus, productions of "peep" and "pop" were excluded from our results.

19. We thank J. Perkell, K. Stevens, R. Held, and P. Sabes for helpful discussions.

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Mass Spectrometric Analysis of the Anaphase-Promoting Complex from Yeast: Identification of a Subunit Related to Cullins

Wolfgang Zachariae, Andrej Shevchenko, Paul D. Andrews, Rafael Ciosk, Marta Galova, Michael J. R. Stark, Matthias Mann,* Kim Nasmyth*

Entry into anaphase and exit from mitosis depend on a ubiquitin–protein ligase complex called the anaphase-promoting complex (APC) or cyclosome. At least 12 different subunits were detected in the purified particle from budding yeast, including the previously identified proteins Apc1p, Cdc16p, Cdc23p, Cdc26p, and Cdc27p. Five additional subunits purified in low nanogram amounts were identified by tandem mass spectrometric sequencing. Apc2p, Apc5p, and the RING-finger protein Apc11p are conserved from yeast to humans. Apc2p is similar to the cullin Cdc53p, which is a subunit of the ubiquitin–protein ligase complex SCF^{Cdc4} required for the initiation of DNA replication.

The APC mediates cell cycle–regulated ubiquitination, and thereby degradation, of proteins containing sequences called destruction boxes (*1–4*). Entry into anaphase depends on the degradation of proteins such as Pds1p and Cut2p, which inhibit sister chromatid separation (*5*, *6*). Degradation of mitotic cyclins inactivates cyclin-dependent kinases (CDKs), which is important for exit from mitosis and is a prerequisite for DNA-replication in the subsequent cell cycle (*7*).

Five subunits of the yeast APC (Apc1p, Cdc16p, Cdc23p, Cdc26p, and Cdc27p) have been identified through genetic analysis (*3*, *8*). However, additional subunits were detected in APC particles purified from yeast and *Xenopus* oocytes (*8*, *9*). Advances in the analysis of proteins by mass spectrometry and sequencing of the entire yeast genome provide a strategy to identify the components of multiprotein complexes that can be biochemically purified (*10*). We used this approach to identify five additional subunits of the APC.

To analyze the composition of the APC, we labeled cells expressing Cdc16p with Myc epitopes (Cdc16-Myc6p) with ^{35}S, and the complex was immunoprecipitated with an antibody to Myc (*11*). Proteins of ~90 (Apc2p) and 70 kD were detected in addition to Apc1p, Cdc16-Myc6p, Cdc27p, and Cdc23p (Fig. 1A). Mass spectrometric analysis revealed that the 70-kD band consists of two proteins, p70 (Apc5p) and p68 (Apc4p). Proteins migrating at ~40 (Apc9p), 32 (Apc10p), 23 (Apc11p), 20 [Cdc26p (*8*)], and 19 kD (Apc13p) were also detected (Fig. 1B). All of these proteins were detected in precipitates from strains expressing different epitope-tagged APC subunits but not from control strains, indicating that the yeast complex contains at least 12 different subunits.

To identify these proteins, we purified the APC from *CDC16-myc6* or *CDC23-myc9* strains (*12*). One-step immunoprecipitations from unfractionated cell extracts yielded enough material to detect individual subunits on silver-stained gels (Fig. 1A).

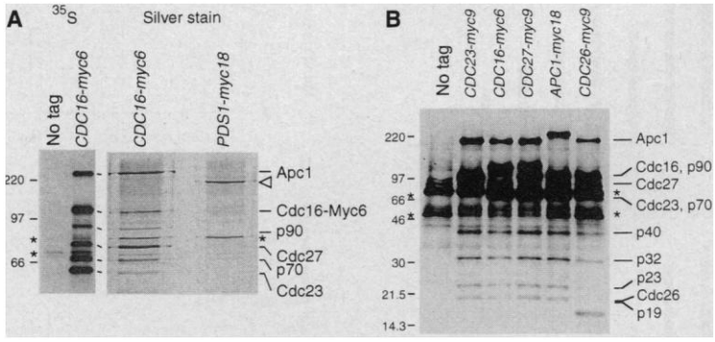

Fig. 1. Subunit composition of the APC. (**A**) Detection and purification of APC subunits. Proteins immunoprecipitated with an antibody to Myc from extracts from *CDC16* (wild type or *PDS1-myc18*) and *CDC16-myc6* cells were separated in SDS-polyacrylamide gels. Proteins from ^{35}S-labeled cells (5×10^7) were detected by fluorography (*11*) (left). For mass spectrometry, immunoprecipitates from 10^{10} cells were detected by silver staining (*12*, *13*) (right). (◁) A protein coimmunoprecipitating with Pds1-Myc18p. Pds1p is stained only weakly. (*) Proteins whose precipitation is not Myc-dependent. (**B**) Small APC subunits. Immunoprecipitates from ^{35}S-labeled cells expressing Myc-tagged APC subunits were separated in a 4 to 20% gradient gel. Molecular sizes are indicated on the left (in kilodaltons).

W. Zachariae, R. Ciosk, M. Galova, K. Nasmyth, Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria.
A. Shevchenko and M. Mann, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69012 Heidelberg, Germany.
P. D. Andrews and M. J. R. Stark, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK.
*To whom correspondence should be addressed.

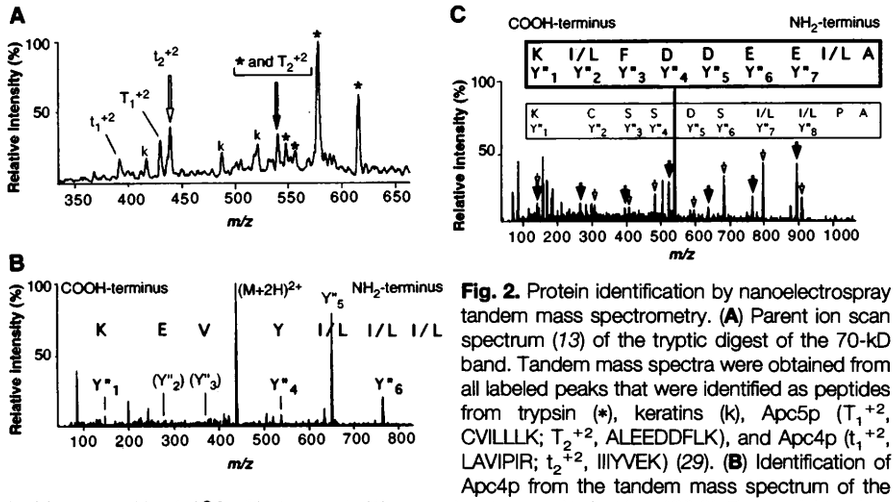

Fig. 2. Protein identification by nanoelectrospray tandem mass spectrometry. (**A**) Parent ion scan spectrum (*13*) of the tryptic digest of the 70-kD band. Tandem mass spectra were obtained from all labeled peaks that were identified as peptides from trypsin (*), keratins (k), Apc5p (T_1^{+2}, CVILLLK; T_2^{+2}, ALEEDDFLK), and Apc4p (t_1^{+2}, LAVIPIR; t_2^{+2}, IIIYVEK) (*29*). (**B**) Identification of Apc4p from the tandem mass spectrum of the doubly charged ion t_2^{+2} [unfilled arrow in (A), mass-to-charge ratio (*m/z*) = 439.2]. The partial tandem mass spectrum above the parent ion was acquired separately (31 scans accumulated) and then combined with the full-scan spectrum (8 scans accumulated). This eliminated the chemical noise in the most informative part of the spectrum. Databases were searched with peptide sequence tags derived from the mass differences between adjacent COOH-terminal ions (Y″ ions) (*30*). After retrieval of the matching sequence, detection of all predicted ions confirmed the identification. (**C**) Identification of Apc5p in the 70-kD band. The tandem mass spectrum of the peak at *m/z* = 539.8 [filled arrow in (A)] yields an ion series (filled arrowheads) from an Apc5p peptide (upper sequence) and another series (unfilled arrowheads) from a trypsin peptide (lower sequence; C, cysteine S-acetamide).

Proteins were identified by nanoelectrospray tandem mass spectrometric sequencing (*13*). The identification of two proteins in the 70-kD band is shown in Fig. 2. Because of the small amount of protein, a normal nanoelectrospray mass spectrum failed to provide clear peptide candidates. However, peptide ions could be distinguished from chemical noise by parent ion scans (*13*) (Fig. 2A). Fragmentation of all peptide ions led to the identification of four peptides from two different yeast proteins (Fig. 2, B and C). One of the peptides was identified in a mixture with a peptide from trypsin (Fig. 2C). The mass spectrometric analysis identified Apc2p, Apc5p, Apc4p, Apc9p, and Apc11p as the gene products of the open reading frames [ORFs (*14*)] YLR127c [853 amino acids (aa)], YOR249c (685 aa), YDR118w (652 aa), YLR102c (265 aa), and YDL008w (165 aa), respectively [Fig. 2 and (*15*)].

We confirmed the identity of these proteins as APC subunits by modifying the endogenous genes to encode epitope-tagged variants (*16*). Immunoprecipitates from

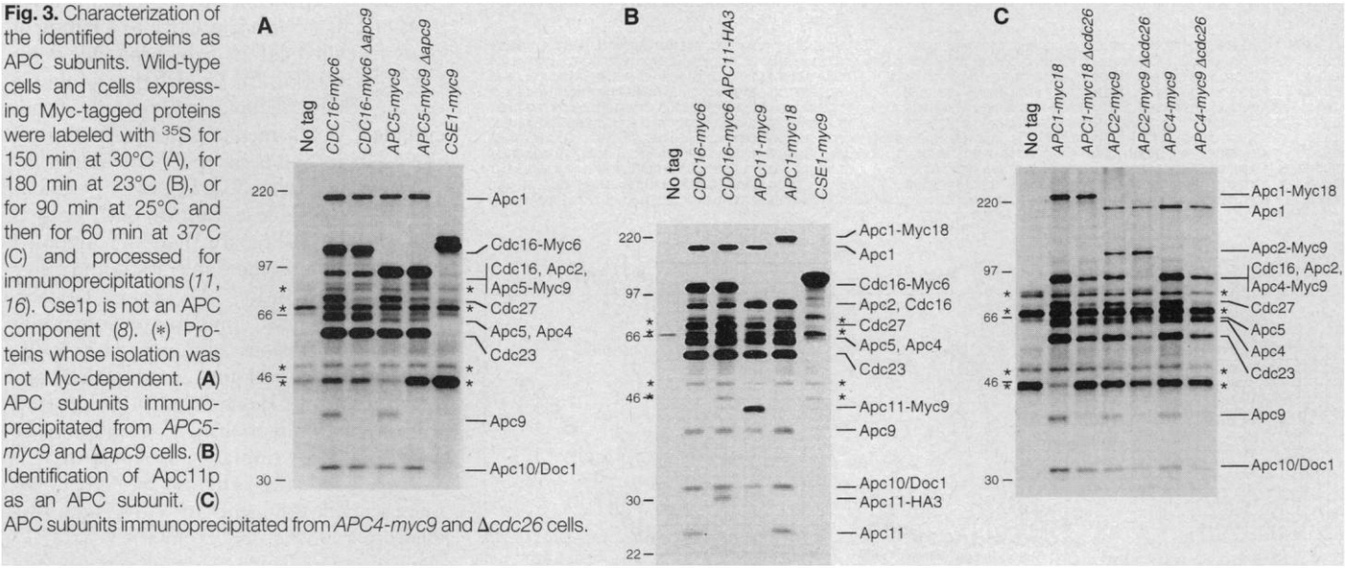

Fig. 3. Characterization of the identified proteins as APC subunits. Wild-type cells and cells expressing Myc-tagged proteins were labeled with ^{35}S for 150 min at 30°C (A), for 180 min at 23°C (B), or for 90 min at 25°C and then for 60 min at 37°C (C) and processed for immunoprecipitations (*11*, *16*). Cse1p is not an APC component (*8*). (*) Proteins whose isolation was not Myc-dependent. (**A**) APC subunits immunoprecipitated from *APC5-myc9* and Δ*apc9* cells. (**B**) Identification of Apc11p as an APC subunit. (**C**) APC subunits immunoprecipitated from *APC4-myc9* and Δ*cdc26* cells.

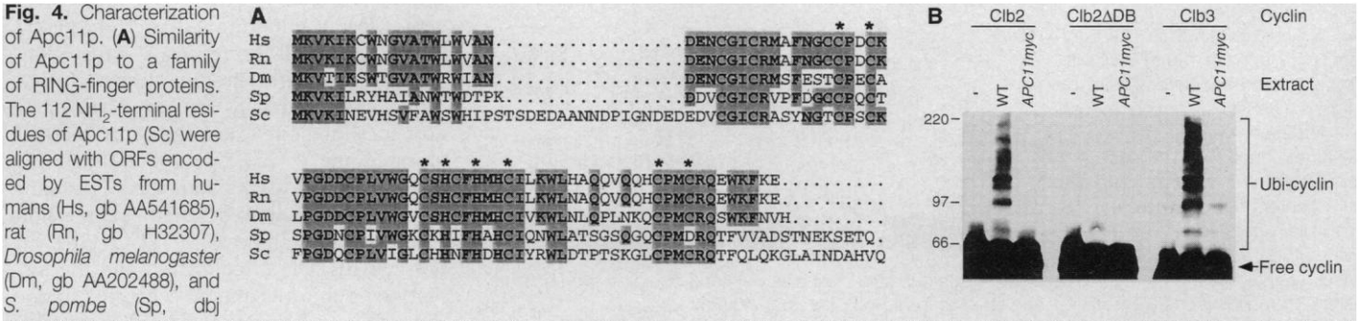

Fig. 4. Characterization of Apc11p. (**A**) Similarity of Apc11p to a family of RING-finger proteins. The 112 NH_2-terminal residues of Apc11p (Sc) were aligned with ORFs encoded by ESTs from humans (Hs, gb AA541685), rat (Rn, gb H32307), *Drosophila melanogaster* (Dm, gb AA202488), and *S. pombe* (Sp, dbj AB001022; an intron was removed) (*19*). (*) Zinc-binding residues conserved in RING-finger proteins (*20*). Residues identical in at least three sequences are shaded (*29*). (**B**) Cyclin ubiquitination in extracts from wild-type (WT) and *APC11-myc9* cells. Strains (*MATa* Δ*pep4* Δ*bar1*) were arrested in G_1 with α-factor at 23°C and shifted to 37°C for 30 min. Extracts were incubated with adenosine 5′-triphosphate and HA3-tagged cyclins (*4*). Cyclin-ubiquitin conjugates were detected by immunoblotting with an antibody to HA. Clb2ΔDBp lacks the destruction box.

APC2-myc9, *APC4-myc9*, *APC5-myc9*, and *APC11-myc9* cells all contained the same set of proteins, which included all known constituents of the yeast APC (Fig. 3). Co-immunoprecipitation of Apc9p with Cdc16-Myc6p or Apc5-Myc9p was dependent on the *APC9* gene (Fig. 3A). Recently, the *DOC1* gene, which encodes a 33-kD protein required for cyclin proteolysis, was identified through genetic analysis (*17*). Immunoprecipitations from ^{35}S-labeled *DOC1-myc9* and *CDC16-myc6 DOC1-HA3* cells revealed that *DOC1* encodes the APC subunit Apc10p (*18*).

One copy of each ORF in diploid strains was replaced by a *HIS* marker (*16*). Tetrad analysis revealed that *APC2*, *APC4*, *APC5*, and *APC11* are all essential genes. His$^+$ spores arrested as large, budded cells within one to three cell divisions after germination.

Haploid cells containing a deletion of *APC9* were viable at 25° and 37°C. However, Cdc27p was largely absent in precipitates from Δ*apc9* strains (Fig. 3A). Apc9p might stabilize the interaction of Cdc27p with the APC. After release of small G_1 cells at 37°C, spindle elongation and sister chromatid separation were delayed by 15 min in Δ*apc9* cells (*18*). Although not an essential gene, *APC9* is required for efficient entry into anaphase. *CDC26* encodes another nonessential APC subunit whose function is only required for growth at increased temperatures (*8*). In immunoprecipitates from Δ*cdc26* strains, the amounts of Cdc16p, Cdc27p, and Apc9p were reduced, whereas the other subunits were still associated with each other [Fig. 3C and (*18*)]. Cdc26p might be required for the incorporation of a set of subunits into the APC, especially at increased temperature.

Database searches (*19*) identified Apc11p as a member of a conserved family of proteins containing a RING finger (Fig. 4A). The RING domain contains two zinc ions, is found in many eukaryotic proteins with diverse functions, and is thought to mediate protein-protein interactions (*20*). Incubation at 37°C of an *APC11-myc9* strain led to the accumulation of cells with short and long spindles, indicating a defect in the onset of anaphase and exit from mitosis. (*18*). Extracts from G_1-arrested *APC11-myc9* cells shifted to 37°C were defective in the ubiquitination of mitotic cyclins (Fig. 4B).

Database searches (*19*) revealed similarity of the COOH-terminal region of Apc2p to a putative ORF from *Caenorhabditis elegans* (K06H7.5) and to a mouse protein whose COOH-terminal 426 amino acids could be assembled from expressed sequence tags (ESTs) [Fig. 5A and (*18*)]. The mouse sequence is 96% identical to that of human Apc2p (*21*). Apc2p contains a region of 180 aa with similarity to a family of proteins called cullins, which include yeast Cdc53p (*22*) (Fig. 5A). Cdc53p is a subunit of the SCF^{Cdc4} ubiquitin–protein ligase complex, which mediates ubiquitination of the CDK inhibitor Sic1p by the ubiquitin-conjugating enzyme Cdc34p (*23*). Degradation of Sic1p is essential for entry into S phase (*24*). Cullins are therefore involved in both chromosome duplication and sister chromatid separation. Apc2p and Cdc53p may interact with components commonly required by various ubiquitin–protein ligases such as ubiquitin-conjugating enzymes. Indeed, Cdc53p binds Cdc34p, and this interaction is abolished by mutations in the region with similarity to Apc2p (*25*).

To analyze the function of Apc2p, we mutagenized the gene in vitro, and two alleles conferring cell cycle arrest at 37°C were integrated into a haploid strain containing a deletion of the genomic *APC2* gene (*26*). We monitored sister chromatid separation in cells expressing a *tet* repressor–green fluorescent protein fusion (tetR-GFP), which binds to an array of *tet* operator sites integrated near the centromere of chromosome V (*27*). We isolated small G_1 cells from wild-type and *apc2-1* mutant strains and followed their progression through the cell cycle at 37°C (*28*). In *apc2-1* cells, DNA replication and the formation of mitotic spindles occurred at the same time (relative to budding) as in wild-type cells. However, most of the mutant cells failed to separate sister chromatids and to elongate their spindles. Cytokinesis and

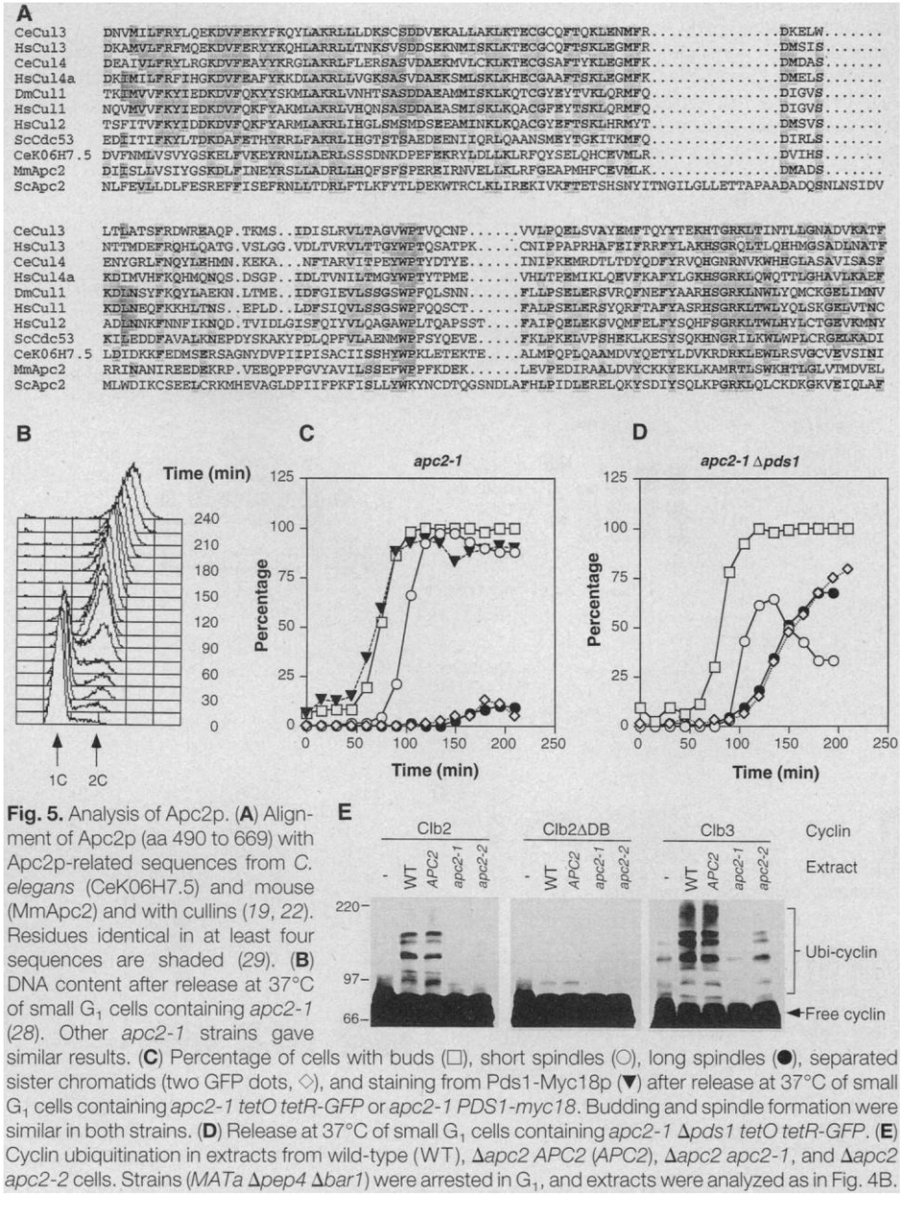

Fig. 5. Analysis of Apc2p. (**A**) Alignment of Apc2p (aa 490 to 669) with Apc2p-related sequences from *C. elegans* (CeK06H7.5) and mouse (MmApc2) and with cullins (*19*, *22*). Residues identical in at least four sequences are shaded (*29*). (**B**) DNA content after release at 37°C of small G_1 cells containing *apc2-1* (*28*). Other *apc2-1* strains gave similar results. (**C**) Percentage of cells with buds (□), short spindles (○), long spindles (●), separated sister chromatids (two GFP dots, ◇), and staining from Pds1-Myc18p (▼) after release at 37°C of small G_1 cells containing *apc2-1 tetO tetR-GFP* or *apc2-1 PDS1-myc18*. Budding and spindle formation were similar in both strains. (**D**) Release at 37°C of small G_1 cells containing *apc2-1 Δpds1 tetO tetR-GFP*. (**E**) Cyclin ubiquitination in extracts from wild-type (WT), Δ*apc2 APC2* (*APC2*), Δ*apc2 apc2-1*, and Δ*apc2 apc2-2* cells. Strains (*MATa Δpep4 Δbar1*) were arrested in G_1, and extracts were analyzed as in Fig. 4B.

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.
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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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31. We thank A. Hyman for bringing together the labs of M.M. and K.N.; A. Schleiffer and B. Habermann for sequence alignments; A. Wach for pFA6a-HIS3MX6; H. Yu, J.-M. Peters, R. King, M. Kirschner, and M. Tyers for communicating results; and M. Glotzer, L. Huber, U. Mühlner, and J.-M. Peters for reading the manuscript.

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Identification of a Cullin Homology Region in a Subunit of the Anaphase-Promoting Complex

Hongtao Yu, Jan-Michael Peters,* Randall W. King, Andrew M. Page, Philip Hieter, Marc W. Kirschner†

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