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Sequential activation and inactivation of cyclin dependent kinases (CDK) regulate eukaryotic cell cycle transitions. The periodicity of CDK activity provides a molecular basis for unidirectional cell cycle progression and is partly controlled by ubiquitin-mediated proteolysis of both cyclins and CDK inhibitors. Deregulated expression of G1 CDK inhibitors and G1 cyclins have been directly linked to breast cancer development. The mechanisms regulating the ubiquitination of these two protein families constitute essential components of G1 cell cycle control in mammalian cells, but they are poorly understood at present. Polyubiquitination of proteins is known to be catalyzed by a cascade of enzymes: E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating). Two issues critical to our understanding of the regulation of protein turnover are how E3 ligases target proteins for ubiquitination and how they themselves are regulated. We have found that cullins partner with another multigene family of evolutionarily conserved proteins, the ROC/APC11 family, to form a complex that contains E3 ubiquitin ligase activity. We are currently investigating whether these complexes can ubiquitinate G1 cyclins and CDK inhibitors and how they are targeted to the ROC/cullin ligase. Understanding these mechanisms could potentially lead to improved diagnostic, prognostic and therapeutic strategies.

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Table of Contents

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July 1999 Annual Summary Report for Award Number DAMD17-98-1-8221

## INTRODUCTION

Sequential activation and inactivation of cyclin dependent kinases (CDK) regulate eukaryotic cell cycle transitions. The periodicity of CDK activity provides a molecular basis for unidirectional cell cycle progression and is controlled, in part, by the ubiquitin-mediated proteolysis of both cyclins and CDK inhibitors. Deregulated expression of G1 CDK inhibitors and G1 cyclins have been directly linked to breast cancer development. The mechanisms regulating the ubiquitination of these two families of proteins constitute essential components of G1 cell cycle control in mammalian cells and are poorly understood at present. These proteins, cyclin D, cyclin E, p27 and p57, are believed to be degraded in a phosphorylation-dependent manner by the ubiquitin-mediated proteasome pathway. This proteolysis pathway is composed of a cascade of enzymes, E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) that catalyze the attachment of ubiquitin to substrates via terminal isopeptide bonds to form polyubiquitinated conjugates. These species of proteins are rapidly recognized and degraded by the 26S proteasome. The E3 ubiquitin ligase is functionally defined to contain two separate activities: the ubiquitin ligase activity to catalyze isopeptide bond formation and the substrate targeting activity. Thus, two issues critical to our understanding of the regulation of protein turnover are how E3 ligases target proteins for ubiquitination and how they themselves are regulated. Cullin-1 represents a multigene family of proteins and has been found to be a component of E3 ligases. In budding yeast, the gene product of CDC53 forms an E3 ligase with the SKP1 protein and F-box containing proteins to mediate the ubiquitin-dependent degradation of the G1 cyclins and G1 CDK inhibitor (called the SCF pathway). Hence, Cdc53p is responsible for regulating the G1-to-S transition in yeast. The purpose of this line of research is to determine how G1 cyclins and G1 CDK inhibitors are regulated by proteolysis during the mammalian cell division cycle. Thus, this research could uncover previously unknown molecular aberrations associated with breast cancer development, potentially leading to improved diagnostic, prognostic, and therapeutic strategies.

BODY

The following is a description of work accomplished pertaining to the Statement of Work for months 1-12.

According to the approved statement of work, Task 1 was to determine if cullin-1 regulated the ubiquitination and proteolysis of cyclin A. Months 1-3 were to be used to determine whether SKP2 mediated cullin-1 interaction with cyclin A. Months 4-8 were to be used to determine if cyclin A ubiquitination is dependent upon association with the cullin-1/SKP1/SKP2 complex. The following is a description of results pertaining to Task1.

Using the yeast two-hybrid system, we have been able to show that human cullin-1, but not human cullin-2, human cullin-3, mouse cullin-4A or human cullin-5, specifically interacts with human SKP1. These results have been confirmed by $^{35}$S-IP of in vitro translated proteins, using the anti-SKP1 antibody that we generated. The SKP1 interaction domain in cullin-1 has been mapped to somewhere within amino acid residues 126-219 in the amino-terminus. Conversely, the cullin-1 interaction domain within SKP1 has been mapped to an amino terminal domain (amino acid residues 1-91). These studies have been performed using the yeast-two-hybrid system. Using western and IP-western techniques, the cullin-1 and SKP1 antibodies that we generated were characterized. Cullin-1 and SKP1 are found in a wide variety of cell and tissue types. Additionally, neither one is post-transcriptionally regulated during the cell cycle. Both are abundant and do not appear to be regulated by their steady state levels as determined by Western analysis using lysates generated from 3T3-L1 cells that have been induced to undergo terminal differentiation into adipocyte cells. Cullin-1 and SKP1 can be found in the same complex in vivo as determined by IP-western experiments using tissue culture cells and using lysates generated from a variety of mouse organs. Furthermore, the cullin-1/SKP1 complex formation is not cell cycle regulated as determined by IP-western experiments in cells that have been synchronized. We have shown that cyclin A is in a complex with cullin-1 and SKP1 as well as SKP-2, which is an F-box protein. This complex is enriched in cells that are in S-phase. No other cyclins were found to be associated with cullin-1. Several of these interactions were confirmed using the baculovirus expression system. These results have led us to suggest that if other cullin members are functioning similarly to cullin-1 in coupling E2 ubiquitin conjugating enzymes to substrates, they may be doing so by utilizing a SKP1- and perhaps F-box-independent pathway(s). The results just summarized were published in the June 1998 issue of *Cell Growth and Differentiation* after receiving notification of ARMY support but prior to the start of funding in July 1998.

Using the baculovirus system we have not been able to show that cyclin A ubiquitination is dependent upon association with cullin-1, SKP1 and SKP2. Though the ubiquitin proteolysis pathway mediates cyclin A degradation, it appears to be performed in mitosis by the E3 ligase called the anaphase promoting complex, or APC. The APC is a complex of proteins that contains a cullin family member, APC2, providing further evidence that cullin-1 is involved in protein degradation. Despite the interaction of cyclin A with the cullin-1 complex, it has become increasingly clear that cyclin A is not a substrate of this complex. Thus, it may be bridging-in associated substrate, for example, E2F transcription factors that are known to be degraded by the ubiquitin proteolysis

pathway, and this is currently under investigation in the lab. This summarizes the work that has gone into completing Task1.

According to the approved statement of work, the first part of Task 2 was to identify cullin members that may interact with cyclins D and E and the CDK inhibitor p27. Months 9-14 were to be used performing the yeast-two-hybrid assay using cullin members as bait and cyclin D, cyclin E and p27 as prey. The following is a description of results pertaining to the relevant timeline for Task 2.

Direct interaction of cullins with cyclins D and E or the CDK inhibitor p27 has not been detected. Expression of human cyclin E in yeast is toxic which complicates the use of the yeast-two-hybrid system for this purpose. In yeast-two-hybrid screens done in the lab using p27 and cyclin D as bait, no cullins were detected to interact. These negative results could be explained by following reasons. First, phosphorylation of substrates has been found to play a major role in the recognition of and targeting to the cullin-1 E3 ligases. Thus, cullins may not interact with a substrate, such as p27, in the yeast-two-hybrid system because it is not properly modified. Second, cullins may or may not be interacting directly with substrate. Thus far, in the case of cullin-1, a SKP1 and F-box pathway is required to interact with substrate. It remains to be seen how other cullins interact with substrate, though the evidence points to a SKP-1 independent targeting pathway. Third, the G1 CKI inhibitor, p27 has been found to be targeted to the cullin-1 E3 ligase via SKP1 and SKP2. Thus, it is possible that cullin-1 regulates all G1 cell cycle protein ubiquitinations while other cullin family members may be important for the degradation of non-cell cycle related proteins. In coupled transfection- IP-western experiments done in our laboratory, cyclin Ds have been detected to specifically interact with ROC1 and ROC2 (see description of ROC proteins below). Since ROC proteins interact tightly with cullins, it follows that cyclin Ds could be found in a complex with cullins. Whether the cyclin Ds are substrates of the ROC/cullin ligases or if cyclin Ds provide a regulatory function specifically for ROC proteins remains to be seen.

Work that has been done ahead of schedule includes characterizing the cullin-3 antibody. The original antibodies that were made against C-terminal peptides for cullin-3 and cullin-4 were not useful. Thus, we generated a new antibody to cullin-3 using an N-terminal peptide. This antibody has been characterized, and it works very well. It is capable of western blotting cullin-3 protein and immunoprecipitating cullin-3 protein. Thus, this antibody will be useful for future experiments. Generation of a new cullin-4 antibody against an N-terminal peptide is underway.

Our laboratory has also cloned a gene named ROC1, for regulator of cullin. This new gene was found in yeast-two-hybrid screens using mouse cullin-4A and cullin-1 as baits. We have been able to show that this protein belongs to a multi-gene family that includes ROC1, ROC2 and APC11. ROC1 and ROC 2 interact with the entire family of cullin proteins with high affinity. APC11 interacts only with APC2, a protein that has limited sequence similarity to the cullins and is a component of the APC which is the E3 ligase active in mitosis. ROC1 is an evolutionarily conserved gene whose budding yeast homolog is 67% identical. I have shown that in yeast, ScROC1 also interacts with the entire family of cullin proteins. We have been able to show that the complexes made

between a ROC family member and a cullin family member contain ubiquitin ligase activity. ROC1 is essential for this activity, and the ROC1/cullin-1/SKP1/β-TrCP (F-box protein) complex is responsible for degrading phosphorylated IκBα. In studies that I have performed using yeast genetic experiments, the following results were obtained. Depletion of ScROC1 results in multiple, elongated buds, a unique phenotype shared with yeast that harbor temperature sensitive mutations in CDC53 (a cullin), CDC34 (an E2 enzyme) and CDC4 (an F-box protein). ScROC1 is required for the degradation of Sic1p and Cln2, a G1 CDK inhibitor and G1 cyclin, respectively. Additionally, expression of human ROC1 and ROC2, but not APC11, can rescue the phenotype of ScRoc1p depleted yeast. Our results demonstrate the functional conservation and specificity among members of the ROC/APC11 family, that ROC/cullin complexes are capable of catalyzing ubiquitin ligations, and that ROC1 is an intrinsic subunit of the ligase activity. Thus, combinations of ROC/APC11 and cullin proteins potentially constitute a wide variety of ubiquitin ligases. During these studies, we have established in our laboratory an in vitro substrate-independent ubiquitination assay, an in vitro substrate-dependent ubiquitination assay, and techniques to perform yeast genetic experiments. The results just summarized were published in the April 1999 issue of *Molecular Cell.*

As this report describes, we have made much progress in understanding the role of the cullin family in regulating proteolysis of cell cycle proteins. As we continue our work in this field, we hope to define the mechanisms targeting mammalian G1 cyclins and G1 CDK inhibitors for regulated proteolysis. Thus, understanding these mechanisms could aid in understanding how some molecular aberrations occur in breast cancer development, and it could allow us to contribute to improved diagnostic, prognostic, and therapeutic strategies for this disease.

Appendix A

Key Research accomplishments

- Characterization of cullin-1 and SKP1 proteins and complex formation
- Completion of Task 1
- Partial completion of Task 2, as scheduled
- Characterization of yeast ScROC1, a cullin binding partner, using yeast genetics
- Establishment of the ROC1/cullin family of ubiquitin ligases in proteolytic control of G1 cell cycle proteins

List of Reportable outcomes

Manuscripts

Michel, J.J., and Xiong, Y. (1998). Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. *Cell Growth and Diff.* 9:435-449.

Ohta, T., Michel, J.J., Schottelius, A., and Xiong, Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Mol. Cell* 3:535-541.

Abstracts

Michel, J.J. and Xiong, Y. Human cullin 1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. The Cell Cycle, May 20-24, 1998. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Michel, J.J., Ohta, T., and Xiong, Y. ROC1, a homologue of APC11, represents a candidate family of cullin ubiquitin ligase regulators. The Molecular Basis of Cancer, March 15-21, 1999. Keystone Symposia, Taos, New Mexico.

Patents

The ROC1 patent has been applied for.

# Human CUL-1, but not Other Cullin Family Members, Selectively Interacts with SKP1 to Form a Complex with SKP2 and Cyclin A[1]

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## Abstract

**The budding yeast gene product, CDC53p, forms E3-like SCF complexes with SKP1 and F-box-containing proteins to mediate the ubiquitin-dependent degradation of G1 cyclins and cyclin-dependent kinase (CDK) inhibitors. Cdc53 represents a multigene family, the human homologues of which, the cullin family, include at least six distinct members. We have found that human cullin 1, but not the other closely related cullins 2, 3, 4A, and 5, selectively interacts with human SKP1. This CUL1-SKP1 interaction is mediated by the $NH_2$-terminal domains of both proteins, and the association appears to be required for the interaction of CUL1 with SKP2, an essential element of the S-phase cyclin A-CDK2 kinase. In an asynchronous population of dividing cells, a minor amount of CUL1 specifically associates with cyclin A but not with other cyclins or CDK inhibitors. The steady-state levels of both CUL1 and SKP1 as well as their association with one another remain relatively constant throughout the cell cycle and in postmitotic cells. Our findings indicate that the SCF pathway, although similarly used by the mammalian cullin 1, is not shared by other cullin members. This implies that most cullins may use a SKP1/F-box-independent pathway to facilitate protein degradation.**

## Introduction

The eukaryotic cell division cycle is controlled by a family of protein serine/threonine kinases known as CDKs,[3] which consist of an activating subunit, a cyclin, and a catalytic subunit, a CDK. Sequential activation and inactivation of CDK enzymatic function constitutes the molecular basis for orderly progression through the cell cycle. The primary positive and negative regulation of CDK activity is mediated by the binding of a cyclin and of a CDK inhibitor, respectively (1–3). In general, the steady-state levels of most CDK proteins remain relatively constant during the cell cycle, whereas the abundance of both cyclins and CDK inhibitors oscillates. Indeed, the prototypical cyclin was first discovered as a protein whose abundance likened to saw-toothed oscillations during early clam embryonic cleavage divisions, an event resulting from intense protein synthesis after fertilization coupled with abrupt disappearance at mitosis (4). Unlike their catalytic CDK partners that appear to be much longer lived proteins, most if not all cyclins are characteristically short-lived proteins. For example, the overall half-lives of mammalian cyclins D1 and E are 20–30 min, whereas their catalytic partners CDK4/6 and CDK2, respectively, are much longer lived proteins, with estimated half-lives of 4 h (5–9). As a result of their short half-lives, cyclins act as rate-limiting factors in CDK complex formation, allowing catalytic CDK subunit recycling and providing an irreversible and rapid deactivation mechanism. Conversely, proteolytic degradation of CDK inhibitors leads to an irreversible release of CDK inhibition. Two distinct families of CDK inhibitors, the p21 family and the p16/INK4 family, have been identified in mammalian cells (3). Although the INK4 inhibitors appear to be stable proteins (10), both p21 and $p27^{KIP1}$, like cyclins, are short-lived proteins with estimated half-lives of 30–60 min (11–13). Together, rapid turnover of cyclin and CDK inhibitor proteins provides cells with the ability to quickly and irreversibly respond to a variety of environmental cues to drive unidirectional cell cycle progression. However, the mechanisms that regulate the degradation of both cyclin and CDK inhibitor proteins are not clear at present.

The short half-lives of most intracellular proteins whose concentrations change promptly as the result of alterations in cellular physiological conditions are conferred by the ubiquitin-dependent proteolytic pathway (14, 15). In brief, this process begins with activation of ubiquitin, a 76-amino acid protein expressed in all eukaryotic cells, in an ATP-dependent manner by an enzyme designated as E1. The charged ubiquitin is then transferred from a thiol site in E1 to a thiol site within an ubiquitin-conjugating enzyme designated as an Ubc or E2. From here, the ubiquitin can either be directly transferred to a side chain amino group of a lysine residue in the substrate or indirectly targeted through an additional step involving an ubiquitin ligase (E3). This series of reactions results in the accumulation of proteins with polyubiquitin chains, and these tagged proteins are rapidly detected and degraded by the 26S proteasome. Because most if not all

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[1] This study was supported by USPHS Grant CA-65572 from the NIH (to Y. X.). Y. X. is a recipient of American Cancer Society Junior Faculty Award and a Pew Scholar in Biomedical Science.
[2] To whom requests for reprints should be addressed, at Department of Biochemistry, University of North Carolina, Chapel Hill, NC 27599-3280.
[3] The abbreviations used are: CDK, cyclin-dependent kinase; APC, anaphase-promoting complex; IP, immunoprecipitation; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; TIGR, The Institute for Genome Research; β-gal, β-galactosidase.

polyubiquitinated proteins are indiscriminately delivered to these large protein complexes for degradation, a critical issue central to our understanding of the regulation of protein turnover is how a specific protein is selectively targeted for ubiquitination.

Elements that control ubiquitination of cyclins and CDK inhibitors involve both *cis*-acting sequences within the destruction substrates as well as *trans*-acting regulatory proteins that target them appropriately for ubiquitination and degradation (14). A conserved nine-amino acid stretch commonly known as the destruction box is located approximately 40–50 residues from the amino termini of both S-phase A-type and mitotic B-type cyclins and has been found to be essential for cyclin ubiquitination (16). Mutation or deletion of the destruction box resulted in indestructible cyclins and, thus, caused arrest in late mitosis in cells from a variety of organisms (17–22). Phosphorylation on specific residues of the yeast and mammalian G1 cyclins, Cln2p (23), cyclin D1 (24), and cyclin E (8, 9), as well as of the CDK inhibitors p27 (25, 26) and Sic1p (27–29), which do not contain destruction boxes, is required for the degradation of these proteins by the ubiquitin-proteasome pathway. The machinery that recognizes and ubiquitinates individual substrates remains largely unknown. At least two distinct pathways, one required for initiating DNA synthesis (the CDC34 pathway) and the other involved in chromosome segregation and exit from mitosis (the anaphase-promoting complex/APC or cyclosome pathway), have been identified to mediate proteolysis of cell cycle control proteins via one or more multisubunit protein complexes [recently reviewed by King *et al.* (14)]. The spectrum of destructible substrates regulated by each pathway and the mechanistic details of how each pathway selectively interacts with its substrates are not clear.

Through yeast genetic analysis, several genes have been identified that appear to play a role in selectively targeting the ubiquitination of cell cycle control proteins including cyclins and CDK inhibitors. In budding yeast, Cdc53p acts in concert with Cdc4p, a WD-40-containing protein (30), Cdc34p, an E2 ubiquitin-conjugating enzyme (31), and Skp1p to control the $G_1$-to-S transition by targeting the phosphorylated CDK inhibitor $p40^{Sic1p}$ (27, 29) and cyclins Cln2p and Clb5p for ubiquitin-mediated proteolysis (23, 32, 33). The biochemical function of the Cdc53p/Cdc34p/Cdc4p complex has been found to require Skp1p (28, 29, 32), which was first identified as an S-phase kinase associated protein because of its association with cyclin A and CDK2 (34, 35) and subsequently as a suppressor of a CDK inhibitor proteolysis defect (32) and as a suppressor of kinetochore protein (36). Sequence analysis of several human and yeast SKP1 binding proteins and mutagenic analysis of a yeast Skp1p binding protein, Cdc4p, identified a conserved structural motif, the F-box, that is responsible for binding to yeast SKP1. The complex of Skp1p, Cdc53p, and the associated F-box protein has been dubbed the SCF (28). Among the many F-box-containing proteins are yeast Cdc4p, Grr1, and human SKP2 (32). Recent evidence has suggested that the F-box-containing protein is the factor that confers substrate specificity to the SCF. Cdc4p assembled with this complex allows specific ubiquitination of phosphorylated Sic1p, whereas association with Grr1p specifically promotes Cln1p and Cln2p destruction (27–29). Searching for fission yeast mutants that failed to arrest upon nutrient starvation identified a gene, POP1, that when mutated results in genomic polyploidy and loss of the polyubiquitinated forms of the CDK inhibitor $p25^{Rum1}$ and of the S-phase initiator Cdc18 (37). Like budding yeast Cdc4p, the Pop1 protein contains WD repeats and an F-box, suggesting that its function may also involve an SCF complex. Independently, two evolutionary conserved WD-repeat containing genes, *fizzy* and *fizzy-related* in Drosophila (38) and *Cdc20* and *Hct1/CDH1* in budding yeast (39, 40), have been genetically linked to the negative regulation of both A- and B-type cyclin levels and other APC substrates. Cdc20 and Cdh1/Hct1 proteins appear to act as rate-limiting, substrate-specific activators of APC-dependent proteolysis because their mutations selectively impaired the degradation of the APC substrates Pds1p and of Clb2 and Ase1, respectively (39, 40). Although these proteins contain WD repeats, they lack F-boxes, suggesting the existence of an F-box/SKP1-independent pathway for substrate-specific proteolysis. Thus, the proteolysis pathways mediated by fizzy/Cdc20 and fizzy-related/Hct1/Cdh1 may share with the Cdc4/Grr1/Pop1-mediated pathway some, but not all, components.

In a screen for mutants with excess postembryonic cell divisions in *Caenorhabditis elegans*, a gene, *cul-1,* was identified, the loss of function of which caused hyperplasia of all tissues as a result of the failure to properly exit from the cell cycle (41), suggesting a negative role for the CUL1 protein in the cell cycle. CUL1 represents an evolutionarily conserved multigene family that includes at least seven members in *C. elegans,* six in humans, and three in budding yeast (33, 41). Although the biochemical properties of the CUL1 protein were unknown at the time it was isolated, it was subsequently discovered that it contained significant sequence homology to yeast Cdc53p (33), implicating members of the cullin family as regulators of the ubiquitin-mediated proteolysis of divergent cellular proteins including cyclins and CDK inhibitors. Very recently, a subunit of the mitotic APC complex, Apc2p, was found to harbor sequence similarity to CDC53/cullins (42, 43), further implicating the cullin proteins in regulating ubiquitin-dependent proteolysis. To elucidate the molecular mechanisms underlying the function of the cullin gene family in mammalian cells, we have undertaken a search for cellular proteins that interact with human CUL1. We report here that human cullin 1 interacts with SKP1 and via SKP1 forms a complex with SKP2 and cyclin A, indicating that the SCF pathway is evolutionarily conserved and similarly used by the mammalian cullin 1. Surprisingly, SKP1 does not interact with other members of the cullin family, suggesting that most cullin proteins may use a SKP1/F-box-independent pathway(s) to mediate protein degradation.

## Results

**Human SKP1 Interacts with Human CUL1.** To search for cellular proteins that could interact with the human CUL1 protein, we used the yeast two-hybrid system (44). The full-length human CUL1 coding sequence was amplified from a HeLa cDNA library and fused in-frame with the yeast Gal4 DNA binding domain. The resulting vector was transformed

into yeast HF7c cells and sequentially transformed with a human HeLa cDNA library (see "Materials and Methods"). Of an estimated $1.3 \times 10^6$ transformants screened, 29 clones were obtained that were positive for both His and β-gal staining. Southern hybridization analysis revealed that 28 of 29 clones corresponded to the same gene (data not shown), and cDNA inserts from nine representative CUL1-interacting clones were amplified by PCR. Sequencing analysis of all nine clones revealed that they encoded human SKP1 (32, 35). The interaction between the CUL1 and SKP1 proteins appeared to be relatively strong as indicated by the growth of the cotransformed HF7c yeast cells in the presence of 5 mM *HIS3* inhibitor 3-amino-1,2,3-triazole (data not shown). All nine SKP1 clones contain the full-length $p19^{SKP1}$ sequence, suggesting that the $NH_2$ terminus of SKP1 may be important for its interaction with CUL1 in yeast cells.

**Isolation and Characterization of Full-Length Mammalian Cullin cDNAs.** Cdc53/cullin represents a multigene family including three distinct genes in yeast, seven in *C. elegans,* and six in humans to date (33, 41). Human CUL1 contains considerable sequence similarity to the other five cullins, with CUL2 being the closest relative. To test whether the other cullin proteins could also interact with SKP1, it was necessary to identify cDNA clones encoding cullin proteins containing complete $NH_2$ termini, because we subsequently determined that the CUL1-SKP1 interaction is mediated by an $NH_2$-terminal domain in CUL1 (see below). All five other human cullin EST cDNA clones reported previously contain complete COOH-terminal sequences but are truncated at their $NH_2$ termini with the exception of CUL5/VACM-1 (41). Therefore, we first isolated cDNA clones encoding the full-length cullin proteins. cDNA clones containing extended 5′ ends of human or mouse cullins 2, 3, 4A, and 5 were identified from the EST database and further characterized by DNA sequencing (see "Materials and Methods"). This analysis revealed that full-length human CUL2 encodes a 745-amino acid protein with a predicted molecular weight of 87,000, and that full-length human CUL3 encodes a 768-amino acid protein predicting a molecular weight of 89,000. Human CUL5/VACM-1 has been reported previously to encode a 780-amino acid protein with a calculated molecular weight of 91,000 (45). The CUL5 cDNA clone we identified and obtained from the EST database contains the $NH_2$ terminus of human CUL5 and a longer 5′ untranslated region (5′ untranslated region) than reported previously but misses 45 amino acids at its COOH terminus (41). These missing residues of CUL5 were restored by PCR to render the full-length sequence of CUL5 (see "Materials and Methods"). Of the six human cullin genes, *CUL4A* and *CUL4B* encode two very closely related proteins sharing 84% identity in the 288-amino acid region available for both proteins (41), suggesting that they may correspond to two recently diverged genes that perform very similar, if not the same, functions. Full-length cDNA clones were not available for either human CUL4A or CUL4B in the current EST database. Instead, a full-length mouse CUL4A EST cDNA clone was identified that predicts a 759-amino acid open reading frame with a calculated molecular weight of 88,000. Given the extremely close relatedness between mouse and human CUL4A proteins (96% identity in the 315-amino acid region available for comparison of both proteins) and the highly conserved function of SKP1 (Ref. 32 and see below), we used the full-length mouse CUL4A when testing for the interaction with human SKP1.

**Human SKP1 Does Not Interact with Other Members of the Cullin Family.** We fused full-length human CUL2, CUL3, mouse CUL4A, and human CUL5 to the yeast *Gal4* DNA binding domain and tested their abilities to interact with human SKP1 by the yeast two-hybrid assay. In contrast to CUL1, neither CUL2, CUL3, CUL4A, nor CUL5 interacted with SKP1 (Fig. 1*A*). The specificity of SKP1 for CUL1 was further tested in an *in vitro* binding assay. Full-length proteins of human CUL1, CUL2, CUL3, and mouse CUL4A and the human CUL5 (missing 45 COOH-terminal amino acids) were *in vitro* translated in rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine. Individually $^{35}$S-labeled CUL proteins were mixed with an equal amount of similarly *in vitro* translated $^{35}$S-labeled SKP1 protein and immunoprecipitated with anti-SKP1 antibody. Of the cullin family members, only a significant amount of CUL1 coimmunoprecipitated with SKP1, again indicating the specificity of SKP1 for CUL1 (Fig. 1*B*). In addition, we have also obtained the same results in coupled cotransfection and immunoprecipitation experiments that, under the conditions where the CUL1 and SKP1 complex can be readily coimmunoprecipitated, we could not detect either CUL3 or CUL4A in anti-SKP1 immunoprecipitates.[4] Although the CUL1-SKP1 interaction itself is not surprising in light of the recently reported association of yeast Cdc53 and yeast SKP1 (28, 29, 32), it appears remarkable that the closest relative of CUL1, CUL2, as well as the remainder of the cullin family does not interact with SKP1. These results suggest that other cullin members, if similarly functioning to facilitate protein degradation by coupling E2 ubiquitin conjugating enzymes to degradation substrates, may be doing so by using a SKP1/F-box-independent pathway.

**CUL1-SKP1 Interaction Is Mediated by the $NH_2$-Terminal Regions of Both Proteins.** The human *CUL1* gene predicts a 776-amino acid protein with a calculated molecular weight of 90,000 (see "Materials and Methods"). To determine the structural basis underlying the specific interaction between CUL1 and SKP1, we mapped the region of CUL1 that is required for its interaction with SKP1. A series of CUL1 deletions from both $NH_2$ and COOH terminals were generated, fused in-frame to the yeast *Gal4* DNA binding domain, and tested for their abilities to interact with SKP1 in yeast cells. A restriction fragment containing the $NH_2$-terminal 249-amino acid residues of CUL1 was sufficient to interact with SKP1 at a strength indistinguishable from that of full-length CUL1 as determined by the two-hybrid assay in yeast cells (Fig. 2*A*). In addition, a further deletion mutant of CUL1 containing an $NH_2$-terminal fragment of 219 amino acids was found to be sufficient for interaction with SKP1 as determined by coimmunoprecipitation in transfected mammalian cells (data not shown). These results indicate that the CUL1-

[4] T. Ohta and Y. Xiong, unpublished observations.

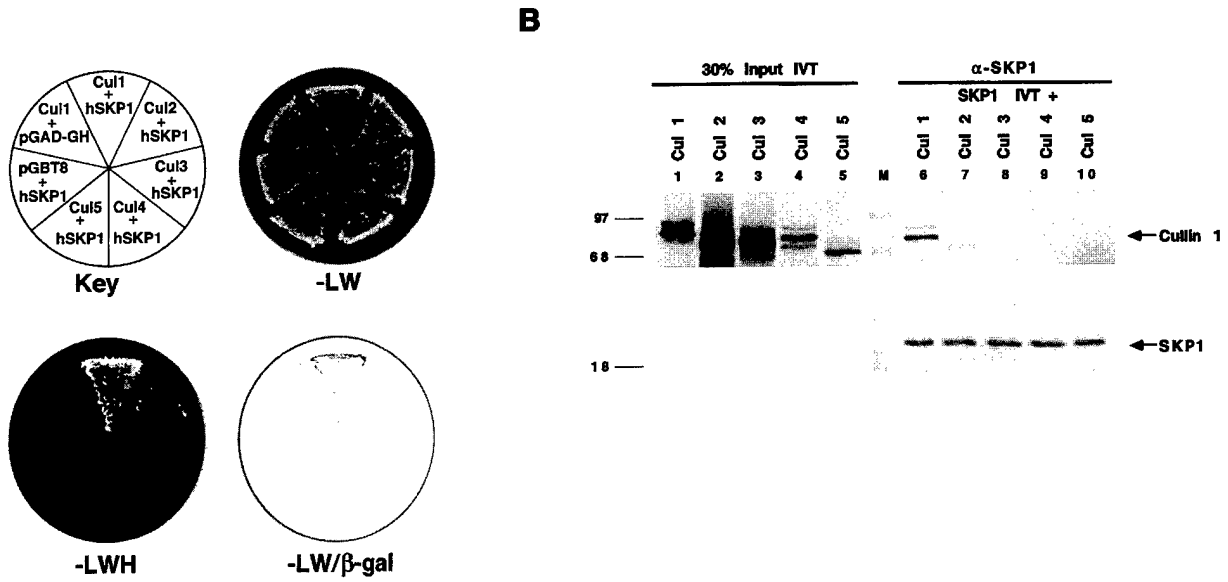



*Fig. 1.* SKP1 specifically interacts with CUL1. *A,* yeast HF7c cells (*his3–200, leu2–3, trp1–901, GAL4 -lacZ, GAL1-HIS3*) containing pGAD-SKP1 or pGAD-GH vector were transformed with either pGBT8-CUL1, pGBT8-CUL2, pGBT8-CUL3, pGBT8-mouse CUL4A, pGBT8-CUL5, or pGBT8 vector. Cells were streaked onto nonselective medium containing histidine (*-LW, top right*) and then replica plated onto selective medium lacking histidine (-LWH, bottom left) to assay for the activation of the histidine reporter gene or streaked onto nonselective medium (*-LW, bottom right*) to assay for β-gal activity. Only CUL1 interacted with SKP1, as determined by growth on a selective -LWH plate and by positive β-gal staining. *B,* [$^{35}$S]methionine-labeled CUL1, CUL2, CUL3, mCUL4A, and CUL5 proteins were individually mixed with an equal amount of [$^{35}$S]methionine-labeled SKP1 and allowed to bind. Mixtures were immunoprecipitated with anti-SKP1 antibody, resolved by denaturing PAGE, and autoradiographed. Only CUL1 was detected in the anti-SKP1 immunoprecipitates.

SKP1 interaction involves a relatively small, $NH_2$-terminal domain of CUL1, and that this interaction is independent of and can be separated from the central and COOH-terminal sequences.

The human *SKP1* gene encodes a 163-amino acid protein with a predicted molecular weight of 19,000. To determine the region of SKP1 required to interact with CUL1, $NH_2$-terminal and COOH-terminal deletion mutants were generated, fused in-frame to the yeast *Gal4* activation domain, and tested for their abilities to interact with CUL1 in yeast cells. The $NH_2$-terminal 91-amino acid residues were sufficient for interaction with CUL1 as determined by the yeast two-hybrid assay (Fig. 2*B*). This evidence, together with the observation that every SKP1 clone identified from the CUL1 yeast two-hybrid library screen was a full-length sequence, indicates that the $NH_2$ terminus of SKP1 mediates its interaction with CUL1.

**SKP2 Interacts with Only the CUL1 Deletions That Retain the SKP1 Binding Site.** SKP1 was first identified as a cyclin A and CDK2-associated protein (34). It was subsequently discovered that binding of SKP1 to cyclin A requires SKP2, an essential element of the S-phase cyclin A-CDK2 kinase, the inhibition of which prevented entry into S phase (35). The finding that CUL1 interacts with SKP1 prompted us to determine whether CUL1 could also interact with SKP2. The cDNA sequence encoding full-length human SKP2 protein was fused in-frame to the GAL4 activation domain and sequentially transformed with full-length CUL1 or the CUL1 deletion mutants (Fig. 3). SKP2 was found to interact with full-length CUL1 in yeast cells and with the CUL1 deletion mutant that retained SKP1 binding activity [CUL1(N219)] but not with the $NH_2$-terminal deletion mutant of CUL1 that lost its binding to SKP1 [CUL1(N126)]. The simplest explanation for these results is that the interaction between CUL1 and SKP2 is mediated by the endogenous yeast SKP1, which shares considerable sequence similarity to (48% identity) and can be functionally complemented by the human SKP1 (32). We have also attempted to map the region in SKP1 that is required for interaction with SKP2 by the yeast two-hybrid assay. Human SKP2 interacted with full-length human SKP1 but not with the $NH_2$ or COOH termini of SKP1, suggesting that SKP2 may be binding to a central sequence of SKP1.

**Ubiquitous Expression of CUL1 and SKP1 Proteins.** To examine the expression and interaction of both CUL1 and SKP1 *in vivo* in mammalian cells, we generated polyclonal antibodies specific to both proteins using synthetic peptides corresponding to the COOH termini of each protein as antigens. The specificities of both antibodies were determined by coupled IP and immunoblotting (IP-Western) experiments. Both antibodies reacted strongly with the denatured recombinant proteins purified from bacteria (Fig. 4, *Lanes M*) and also precipitated from cultured HeLa cells proteins that, based on their comigration with purified recombinant proteins and competition by the antigen peptides, correspond to their respective antigenic proteins (Fig. 4*A*). Whereas the anti-SKP1 antibody was capable of immunoprecipitating SKP1 protein from HeLa cells lysed by a nonionic detergent (0.5% NP40), the anti-CUL1 antibody was able to precipitate CUL1 protein only when cells were lysed by a strong denaturing ionic detergent (*e.g.,* 0.1% SDS). The anti-SKP1 anti-

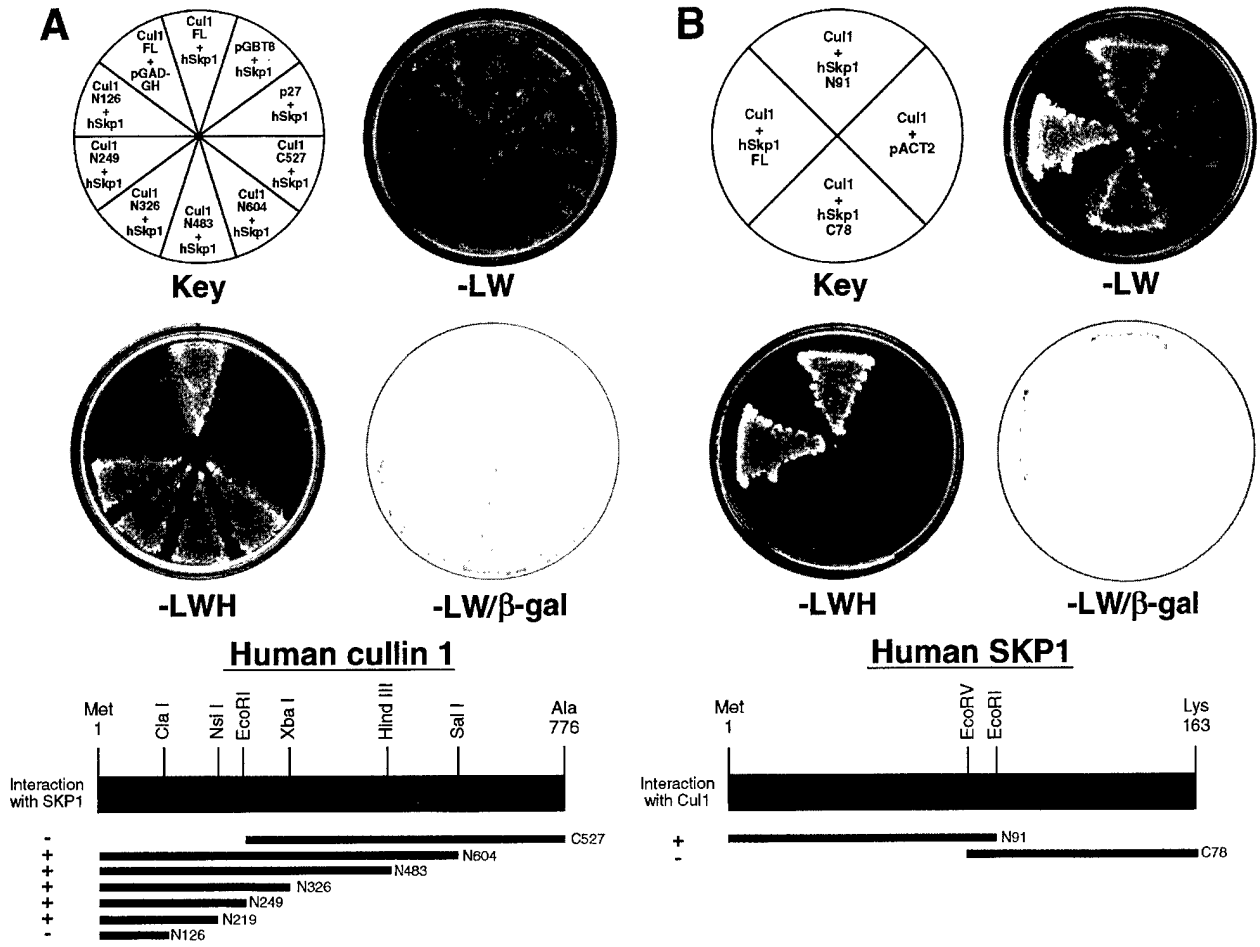



*Fig. 2.* Both CUL1 and SKP1 associate with one another through their $NH_2$ termini. *A*, HF7c yeast cells containing pGAD-SKP1 or pGAD-GH vector were transformed with either pGBT8-CUL1, pGBT8-p27 (as an additional negative control), pGBT8-CUL1(C527), pGBT8-CUL1(N604), pGBT8-CUL1(N483), pGBT8-CUL1(N326), pGBT8-CUL1(N249), pGBT8-CUL1(N126), or pGBT8 vector. Interaction between SKP1 and different CUL1 deletion mutants was assayed as described in Fig. 1. The $NH_2$-terminal 249 amino acids of CUL1 was sufficient for interaction with SKP1. *B*, HF7c yeast cells containing pGBT8-CUL1 were transformed with either pACT2, pACT2-hSKP1 full length, pACT2-hSKP1(N91), or pACT2-hSKP1(C78). Interactions between CUL1 and the different SKP1 deletion mutants were assayed as described in Fig. 1. The $NH_2$-terminal 91-amino acid residues were sufficient for interaction with CUL1.

body was also able to coimmunoprecipitate SKP1-associated CUL1 from HeLa cells lysed by the NP40 buffer (see Fig. 5 below), indicating that the CUL1 protein is released under nondenaturing conditions. In the same NP40 buffer, the anti-CUL1 antibody is able to precipitate CUL1 protein synthesized in rabbit reticulocyte lysate, indicating that failure to precipitate the CUL1 protein *in vivo* using the NP40 lysis buffer is not likely to be the result of an internal folding of the COOH terminus, which would cause the epitope to be inaccessible to the antibody. These observations suggest that the COOH terminus of human CUL1 may be tightly bound by a cellular protein(s), masking the epitope, the association of which with CUL1 is resistant to the NP40 detergent and can only be dissociated by ionic detergent such as SDS. Based on the results obtained from the yeast-two-hybrid CUL1 deletion experiment (Fig. 2*A*), the putative COOH-terminal CUL1 binding protein(s) is not SKP1. Together, these observations suggest that a distinct protein(s), if truly existent, may physically bind to the COOH terminus of CUL1.

Using the antibodies specific to CUL1 and SKP1, we examined the expression level of both proteins in several established human or mouse cells lines derived from five different cell types: lung (IMR-90, WI-38, and VA13) and embryo (NIH3T3) fibroblasts, cervical epithelial cells (HeLa), peripheral T (CEM) and B lymphoblastoid (DG75), osteoblasts (MG63), and preadipocytes (3T3-L1). Both CUL1 and SKP1 proteins are expressed in all cells, suggesting that both proteins are ubiquitously expressed in different tissues and cell types (Fig. 4*B*). In normal human fibroblast cells, many cyclin and CDK proteins, including cyclin A and CDK2, exist predominantly in a quaternary complex with the CDK inhibitor p21 and with the proliferating cell nuclear antigen (34, 46). Viral transformation causes p21 and proliferating cell nuclear antigen to be replaced by SKP1 in cyclin A/CDK2 complexes, partly because of an elevation of $p45^{SKP2}$, a gene that encodes an F-box-containing protein required for the binding of SKP1 to cyclin A and CDK2 (35). The steady-state levels of the CUL1 and SKP1 proteins are similar in one pair

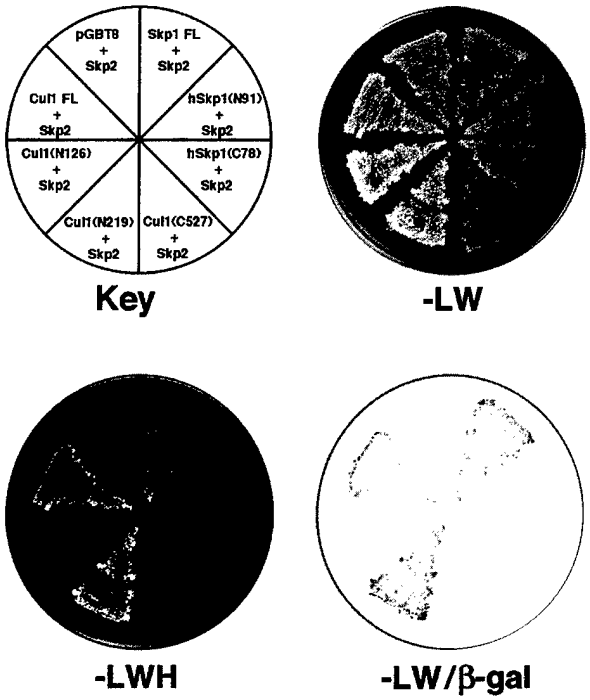



*Fig. 3.* SKP2 interacts with CUL1 only when the SKP1 binding site is present. HF7c yeast cells containing pACT2-SKP2 were transformed with either pGBT8-CUL1FL, pGBT8-CUL1(N126), pGBT8-CUL1(N219), pGBT8-CUL1(C527), pGBT8-SKP1FL, pGBT8-SKP1(N91), pGBT8-(C78), or pGBT8 vector. Interactions between SKP2 and the various CUL1 and SKP1 proteins were assayed as described in Fig. 1. The $NH_2$-terminal 219 amino acids of CUL1 was required and sufficient for interaction with SKP2, an interaction that is likely mediated by yeast SKP1. Only full-length SKP1 interacted with SKP2, indicating that the central sequences of SKP1 may be important for its binding.

of matched normal (WI-38; Fig. 4*B*) and virally transformed lung fibroblasts (VA-13) as well as several other normal/untransformed (IMR-90, NIH-3T3, and 3T3-L1) and transformed cells (HeLa). Consistently, the levels of both CUL1 and SKP1 proteins are similar in 3T3-L1 adipoblasts and 3T3-L1 cells expressing individually type 16 papilloma viral oncoprotein E6 or E7 (data not shown). These observations indicate that unlike SKP2, the expression of neither CUL1 nor SKP1 is affected by the inactivation of p53 and/or pRb function caused by viral transformation. Unexpectedly, both the CUL1 and the SKP1 antibodies, which were raised against COOH-terminal peptides of the human proteins, reacted strongly with proteins in insect Sf9 cells, which judged by antigenicity and size, appear to correspond to insect CUL1 and SKP1 proteins. This observation indicates a high degree of evolutionary conservation of both proteins and also cautions the interpretation of any protein complex reconstitution experiments using the baculovirus expression system.

***In Vivo* Association of CUL1 and SKP1.** Identification of the CUL1 and SKP1 interaction by the yeast two-hybrid assay prompted us to seek evidence for their *in vivo* association in mammalian cells. Total cell lysate was prepared from asynchronously growing HeLa cells and immunoprecipitated with antibody specific to either SKP1 or CUL1. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-CUL1 and anti-SKP1 antibodies. The anti-SKP1 immunocomplex contained a $M_r$ 90,000 protein that was competed off by the SKP1 antigen peptide which, when immunoblotted, was recognized by the CUL1 antibody (Fig. 5, *Lanes 5* and *6*), demonstrating a specific *in vivo* association of CUL1 with SKP1. Reciprocal immunoprecipitation using the anti-CUL1 antibody only precipitated a trace amount of SKP1 due to the use of strong ionic detergent as required for the anti-CUL1 precipitation (longer exposure of Fig. 5, *Lane 4*).

**CUL1-SKP1 Association Remains Constant during the Cell Cycle.** We next determined whether the CUL1-SKP1 association oscillated during the cell. HeLa cells were arrested in S phase by a reversible DNA polymerase inhibitor, aphidicolin. They were released and allowed to progress through $G_2$ phase, mitosis, and the next $G_1$ phase. Cell lysates prepared from different time points after release of the aphidicolin block were immunoprecipitated with the anti-SKP1 antibody, and the steady-state levels of SKP1 and its association with CUL1 were determined by IP-Western. Cell cycle progression after release from the S-phase aphidicolin block was monitored by FACS analysis. Both the level of SKP1 and of its associated CUL1 remain constant as the cells traverse S and $G_2$-M phases (Fig. 6). The levels of both SKP1 and SKP1-associated CUL1 protein continue to remain unchanged at later time points when most cells should have exited from mitosis and entered $G_1$ phase (Fig. 6: 11-, 12- and 13-h time points). As the cell population began to lose its synchrony at later time points, the observation that both SKP1 and the SKP1-CUL1 association remain constant in cells entering the next $G_1$ phase should be interpreted with caution.

**CUL1 and SKP1 Expression in Postmitotic Cells.** During terminal differentiation, many cell cycle regulatory proteins, particularly S-phase cyclin A and mitotic cyclin B1, are down-regulated. Preventing accumulation of mitotic cyclin proteins, and thus their associated CDK activities, may be required for cell cycle exit and/or maintaining a stable cell cycle arrest during terminal cell differentiation (38, 41). Cells lacking CUL1 function in *C. elegans cul-1* mutant larvae divide normally but cannot exit normally from the cell cycle during development, thus they undergo excessive divisions and produce abnormally small cells (41). To determine whether human CUL1 and SKP1 may have a similar function, we determined the expression of both proteins and their association during terminal cell differentiation and in postmitotic cells. We chose the mouse preadipocyte cell line 3T3-L1 for this analysis because of its unique cell cycle pattern during terminal adipocyte differentiation and because both CUL1 and SKP1 proteins are expressed in 3T3-L1 cells (Fig. 7*A*). 3T3-L1 preadipocyte cells can be maintained as proliferating cells and can be temporarily arrested in a quiescent state upon confluence. Density-arrested 3T3-L1 cells can be induced to exit from quiescence by the appropriate combination of mitogenic and adipogenic signals to synchronously progress through a required mitotic clonal expansion phase (between day 1 and day 2). After 3 days of differentiation induction, 3T3-L1 cells enter into a

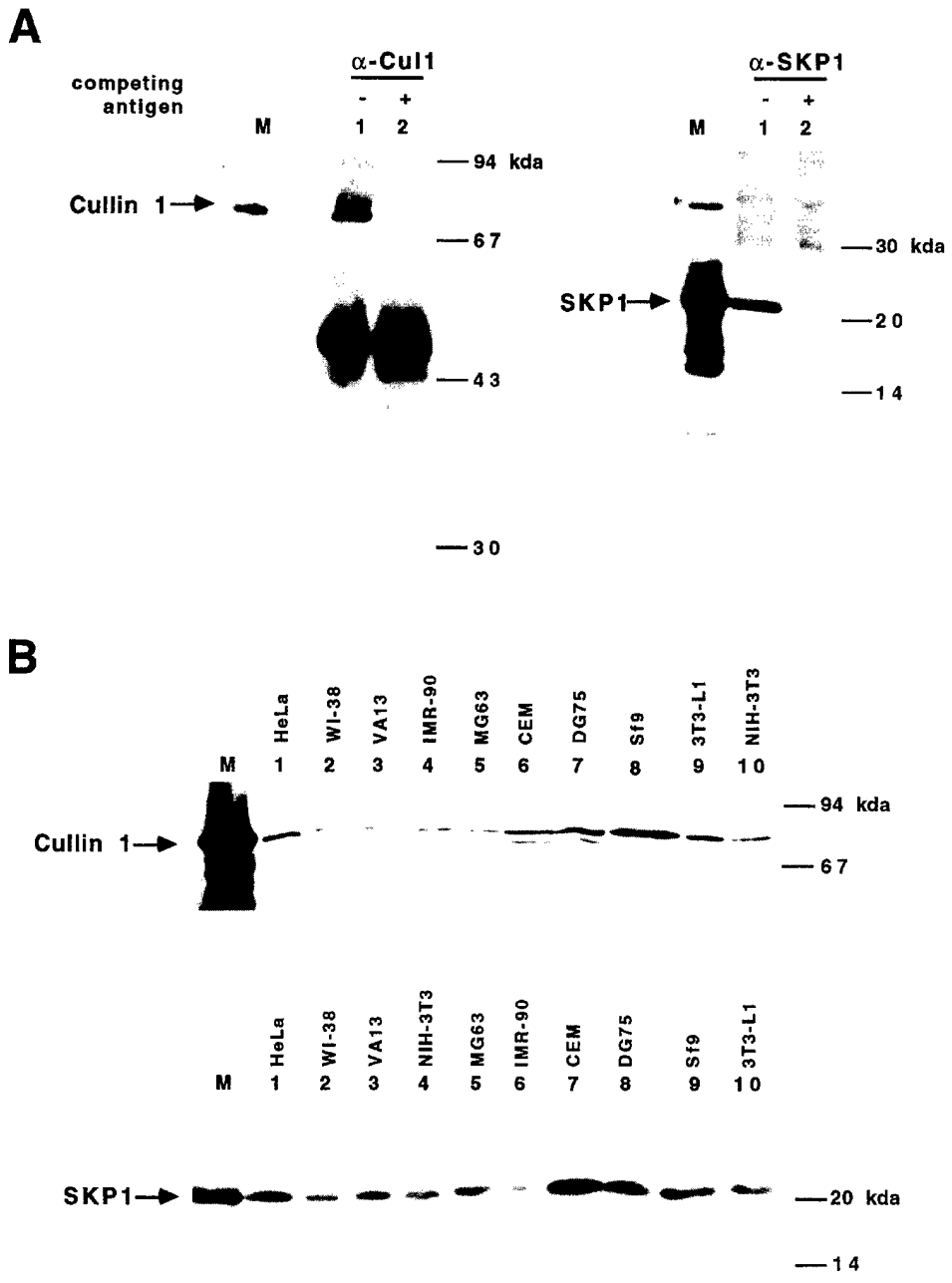



*Fig. 4.* Ubiquitous expression of CUL1 and SKP1 proteins. *A,* characterization of anti-CUL1 and anti-SKP1 antibodies. HeLa cell lysates were immunoprecipitated with the indicated affinity-purified polyclonal antibodies in the presence and absence of the appropriate competing peptide antigen as indicated. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and Western blotted. Bacterial lysates containing recombinant CUL1 and SKP1 proteins were used as positive controls (*Lanes M*). Cells were lysed in either buffer containing SDS (for anti-CUL1 immunoprecipitation) or NP40 lysis buffer (for anti-SKP1 immunoprecipitation). *B,* expression of CUL1 and SKP1 proteins in different cell types. Equal amounts of total lysate were prepared from 10 different cell lines, and the steady-state levels of CUL1 and SKP1 were determined by Western blot analysis.

permanent state of cell cycle arrest as terminally differentiated adipocytes (reviewed in Refs. 47 and 48; also see "Materials and Methods"). As a control (uninduced), a separate set of density-arrested 3T3-L1 cells were similarly switched into a media containing the same mitogenic signal (10% FBS) but lacking hormone inducer. Although a small fraction of cells in the uninduced population was stimulated to enter the cell cycle as indicated by the synthesis of a low level of cyclin A (Fig. 7*A*), the majority of the control cells did not accumulate cytoplasmic triglyceride droplets (data not shown) and remained in $G_0$ quiescence as confirmed by the diminish of cyclin A level almost undetectable level after 2

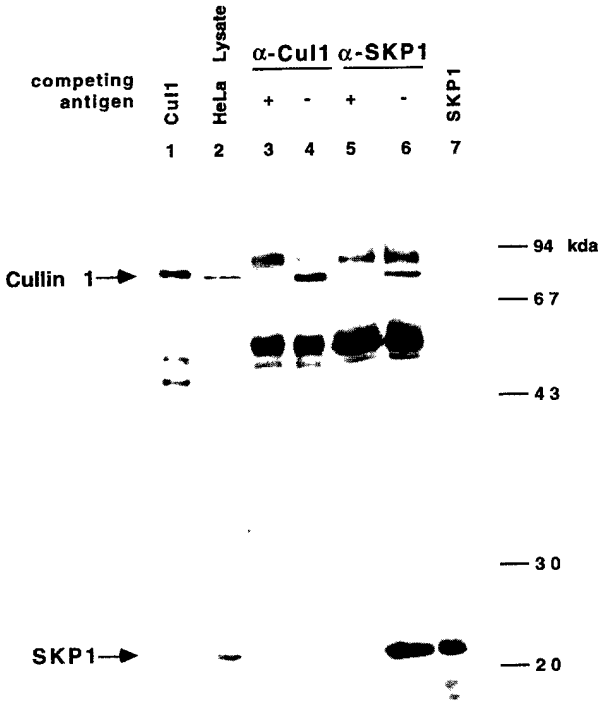



*Fig. 5. In vivo* association of CUL1 and SKP1. HeLa cell lysates were immunoprecipitated with the indicated antibodies in the presence or absence of competing peptide antigen. The immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-CUL1 (*top panel*) or anti-SKP1 (*bottom panel*) antibodies.

days of differentiation induction (Fig. 7*A*), Both CUL1 and SKP1 proteins were readily detected at a level similar to that seen in proliferating 3T3-L1 cells (Fig. 7*A, Lane 1*). As 3T3-L1 cells were hormonally induced to undergo adipogenic differentiation, the steady-state levels of both CUL1 and SKP1 remained essentially unchanged between temporarily arrested, mitotically dividing, and postmitotic cells. Thus, both CUL1 and SKP1 proteins are expressed not only in dividing cells but also in terminally differentiating and permanently arrested cells.

We further determined the state of association between CUL1 and SKP1 in postmitotic cells of several tissues. Total cell lysates were prepared from a collection of adult mouse tissues, and the levels of SKP1 and the SKP1-CUL1 complex were examined by IP-Western analysis. In all varieties of postmitotic tissues examined, the SKP1 protein was abundantly expressed, and the CUL1 protein was found to be associated with SKP1 (Fig. 7*B*). We do not know, however, whether the total amount of CUL1 in these tissues is in association with SKP1 because we have not performed any immunodepletion experiments.

**CUL1 Is Specifically Associated with Cyclin A.** SKP1 was initially identified by its association with cyclin A (34). The finding of the CUL1-SKP1 interaction prompted us to determine whether CUL1 is associated with cyclin A *in vivo*. Cell lysate from asynchronously growing HeLa cells was immunoprecipitated with a polyclonal antibody specific to human cyclin A and probed with antibodies specific to cyclin A, SKP1, and CUL1. CUL1 and SKP1 were both detected in the anti-cyclin A immunocomplex, demonstrating an *in vivo* association of CUL1 with cyclin A (Fig. 8*A, Lane 1*). The amount of CUL1 detected in the anti-cyclin A immunocomplex is considerably less than that seen by anti-SKP1 immunoprecipitation, suggesting that although the majority of CUL1 in the cell is associated with SKP1, only a fraction of CUL1 is associated with cyclin A (Fig. 8*A*, compare *Lanes 1* and *2, top panel*).

Finding the CUL1-cyclin A-SKP1 association *in vivo* led us to ask whether SKP2 was also part of the cyclin A immunocomplex. HeLa cell lysates were harvested from asynchronously growing cells or from cells arrested by aphidicolin in S phase, where SKP2 is accumulated at its highest level (35). Immunoblotting equal amounts of both asynchronous and S-phase lysates showed that both cyclin A and SKP2, but not CUL1 and SKP1, accumulate during S phase (Fig. 8*B, Lanes 1* and *2*). Immunoblotting of anti-cyclin A immunoprecipitates derived from these two cell lysates showed a clear increase in the amount of CUL1, SKP2, and SKP1 associated with cyclin A during S phase as compared with asynchronous cells (Fig. 8*B, Lanes 3* and *4*). We noted that the difference in immunoprecipitated cyclin A from S phase and asynchronous lysates is not proportional to the difference as detected by immunoblotting of the same two cell lysates (Fig. 8, compare *Lanes 3* and *4, Lanes 1* and *2*), suggesting that the amount of cyclin A antibody used in immunoprecipitation is limiting. Thus, we are probably underestimating the increase in the levels of cyclin A-associated CUL1, SKP2, and SKP1 during this S-phase block.

**CUL1 Does Not Associate with Other Cyclin and CDK Complexes.** Finding the CUL1-cyclin A association led us to determine whether CUL1 is also associated with other cyclin proteins, especially mitotic cyclin B1 which, of all of the cyclins, contains the highest sequence similarity to cyclin A including the $NH_2$-terminally located cyclin destruction box known to be essential for cyclin proteolysis. HeLa cell lysate was immunoprecipitated with a battery of antibodies specific to several human cyclin proteins, including cyclin E, cyclin D1, and cyclin B1, and the anti-cyclin immunoprecipitates were immunoblotted with the anti-CUL1 antibody. CUL1 protein was detected in the anti-cyclin A immunoprecipitate but not in the other three cyclin immunocomplexes, cyclin B1, cyclin E, and cyclin D1 (Fig. 8*C, top panel*). All three cyclin antibodies were capable of coimmunoprecipitating their respective antigen protein complexes (34), as further confirmed by the coprecipitation of their respective CDK partners (Fig. 8*C, bottom panel*) with the exception of cyclin D. Cyclin D is expressed at very low levels in HeLa cells, which accounts for the lack of CDK4 in the immunocomplex. We have confirmed the negative association between cyclin D and CUL1 in MG63 cells, which express an abundant amount of cyclin D (data not shown). Similarly, in numerous analyses of $^{35}S$-labeled immunoprecipitates derived from HeLa cells under conditions allowing the detection of the cyclin A-SKP1 complex, we did not observe SKP1 in anti-

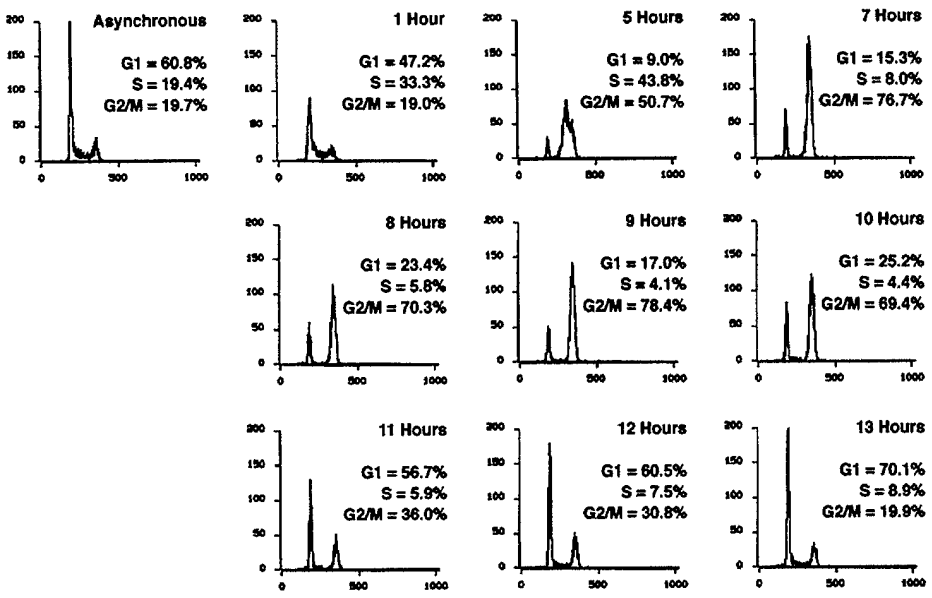
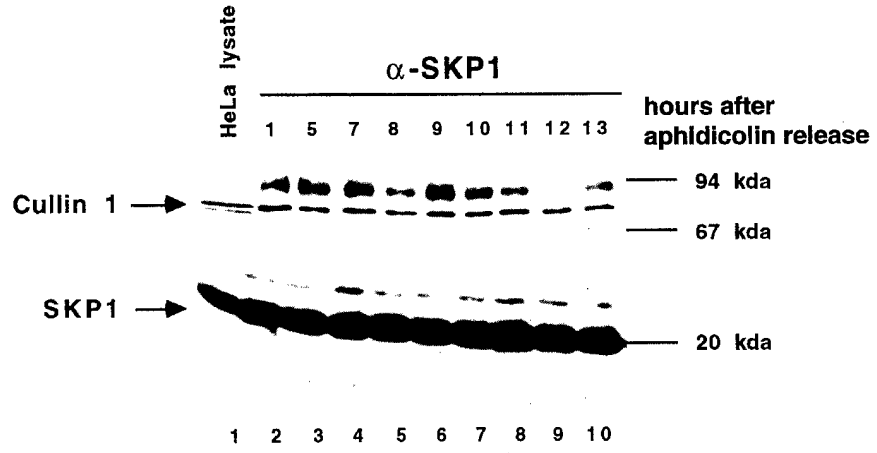



*Fig. 6.* Association of the CUL1-SKP1 complex during the cell cycle. HeLa cells were arrested in S phase by aphidicolin, a reversible DNA polymerase inhibitor. Cell cycle progression after release from the aphidicolin block was monitored by FACS analysis. Cell lysates prepared at different points in time after the aphidicolin block release were immunoprecipitated with the anti-SKP1 antibody, and the steady-state level of SKP1 and its association with CUL1 was determined by Western analysis.




cyclin C, anti-cyclin G1, or anti-cyclin H immunocomplexes.[5] Additionally, whereas we were able to detect CUL1 in the CDK2 immunocomplex,[6] we failed to detect CUL1 in several CDK (CDC2 and CDK4) and CDK inhibitor (p21 and $p27^{KIP1}$) immunocomplexes.[7] We have not determined whether cyclin F, the only cyclin that can directly interact with SKP1 through a structural motif not found in other cyclins, called the F-box (32), associates with CUL1 due to the lack of suitable immunological reagents to cyclin F. From these observations, we conclude that CUL1 preferentially, if not exclusively, interacts with cyclin A.

[5] A. Yee, Y. Zhang, and Y. Xiong, unpublished observations.
[6] Y. Zhang and Y. Xiong, unpublished observations.
[7] J. Michel and Y. Xiong, unpublished observations.

## Discussion

Cullin 1 is a member of a conserved multigene family the function of which has recently been linked to ubiquitin-mediated proteolysis. Cdc53p, the yeast homologue of human cullin 1, is involved in the ubiquitin-mediated degradation of several key cell cycle control proteins, including both the CDK inhibitor Sic1p and the G1 cyclins Clns (27–29). In this report, we present evidence that human cullin 1 specifically interacts with cyclin A and that this CUL1-cyclin A interaction is most likely mediated by SKP1 and SKP2. After the submission of this manuscript, Listwan *et al.* (49) reported similar findings that CUL1 forms a complex with SKP2 and cyclin A as well as the CDC34 ubiquitin-conjugating enzyme. Our studies on the formation of the CUL1-SKP1-SKP2-cyclin A complex *in vivo* are consistent with and confirm the findings

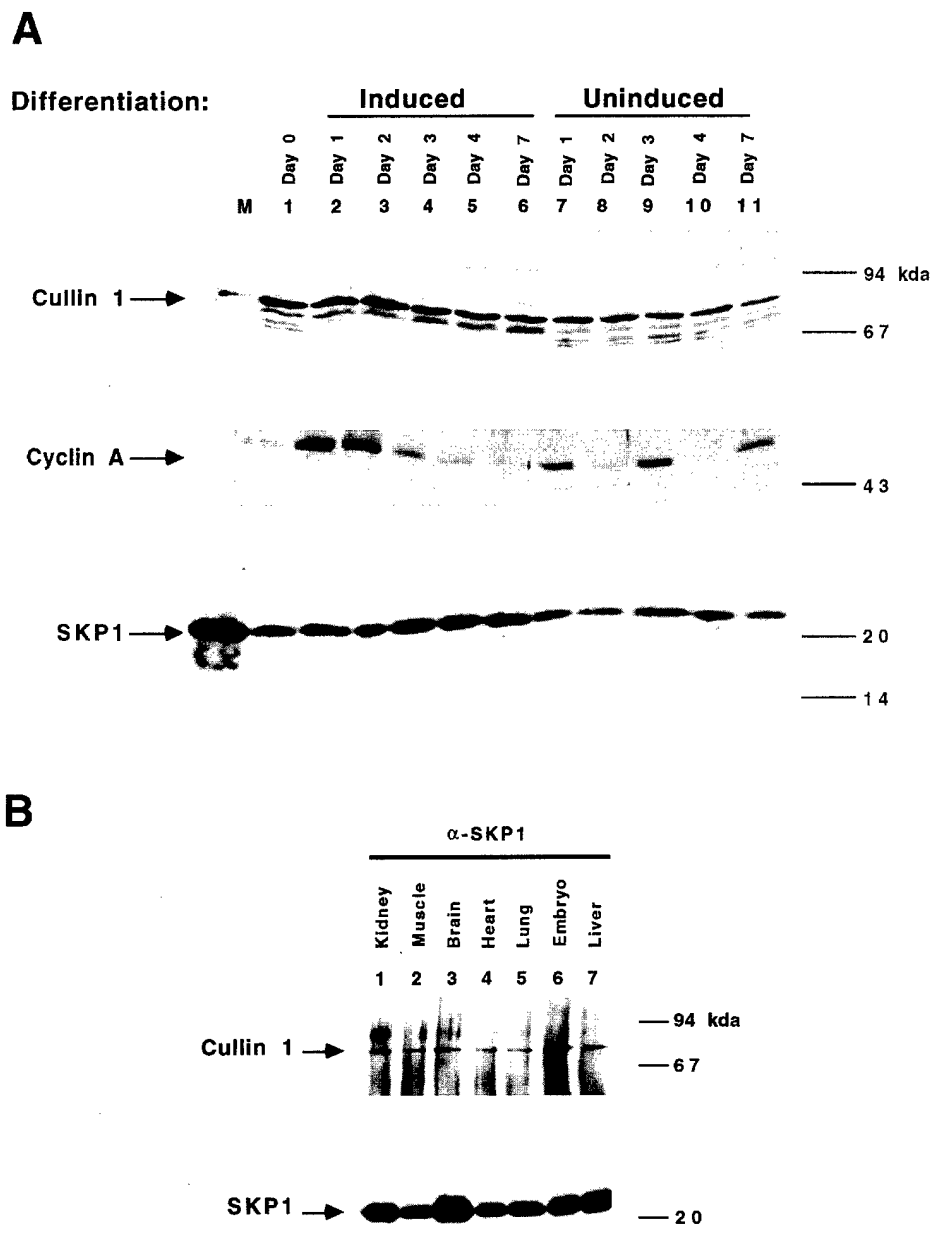
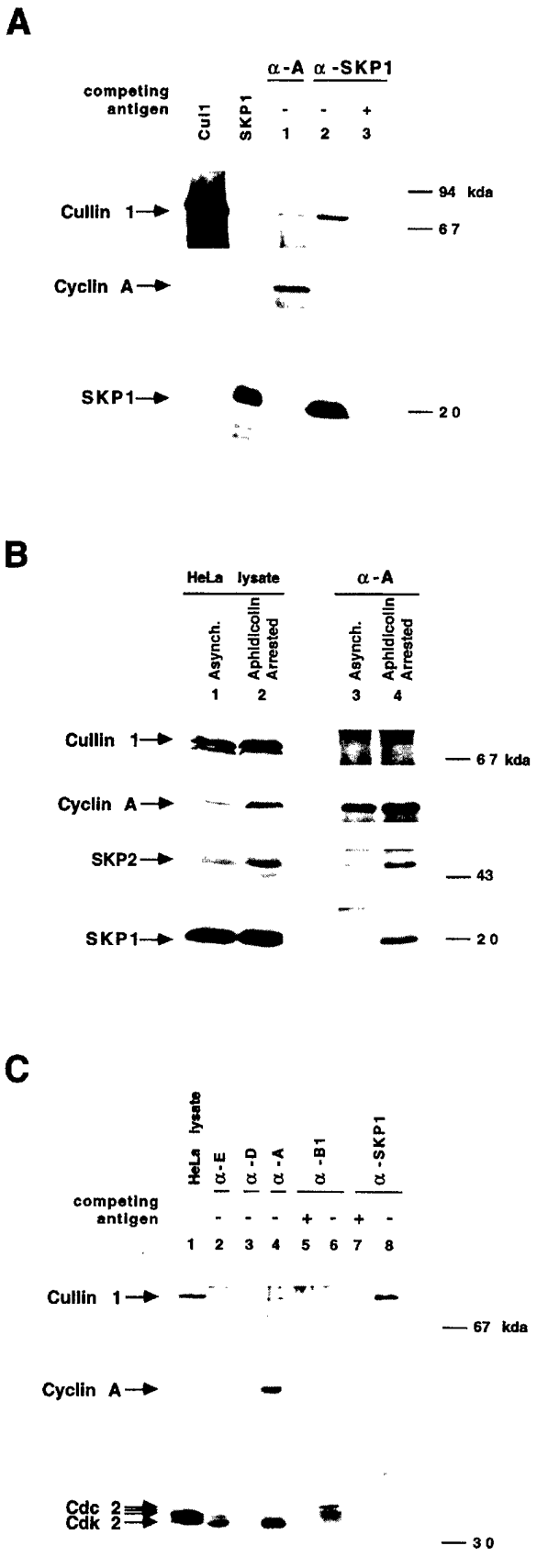



*Fig. 7.* CUL1-SKP1 complexes persist in postmitotic cells. *A*, expression of CUL1 and SKP1 proteins during *in vitro* terminal adipocyte differentiation. Cell lysates prepared from 3T3-L1 cells at different time points during induction of differentiation or from uninduced 3T3-L1 cells were resolved by SDS-PAGE, transferred to nitrocellulose, and Western blotted using antibodies to CUL1, cyclin A, and SKP1. *B*, CUL1-SKP1 complex *in vivo*. Cell lysates prepared from homogenized tissues dissected from a wild-type mouse were immunoprecipitated with anti-SKP1 antibody. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and Western blotted using antibodies to CUL1 (*top*) and SKP1 (*bottom*).

made by Listwan *et al.* (49). We further extend these findings by providing evidence that CUL1 directly interacts with SKP1 and that SKP1 likely functions to mediate the interaction of CUL1 with SKP2. Our findings are consistent with a recent report showing that yeast CDC53 interacts directly with SKP1 and that Cdc53-F-box protein (*e.g.*, Cdc4 and Met 30) interactions are likely bridged by SKP1 (50). We also show that both the expression and complex formation of CUL1 and SKP1 remain unchanged during the cell cycle and persist in stably arrested postmitotic cells, implicating a function for CUL1 and SKP1 in both mitotically dividing and arrested cells. Significantly, we found that SKP1 selectively interacts with only CUL1 but not other members of the cullin family. This finding indicates that the initially identified SCF pathway is used by perhaps only a small number of proteins, the ubiquitin-dependent proteolysis of which requires cullin 1 as a part of the E3 ligase. This finding implies that the ubiquitin-dependent proteolysis of other proteins that similarly require the presence of other cullins in the E3 may involve a SKP1- and F-box-independent targeting pathway(s).

Three lines of evidence collectively suggest that the CUL1-cyclin A interaction is mediated by direct binding in the sequential order of CUL1-SKP1-SKP2-cyclin A: (*a*) CUL1 and SKP1 specifically and directly interact with one another through $NH_2$-terminal domains (Fig. 2); (*b*) the CUL1-SKP2 interaction is most likely mediated by SKP1. This was dem-




onstrated by the precise overlap of the CUL1 binding region for both SKP2 and SKP1 (Fig. 3). In addition, SKP2 is unable to interact with three other cullins that we have tested, CUL 2, CUL3, and mCUL4A.[7] Because SKP1 only binds to CUL1, the negative interactions between SKP2 and the other cullins further reinforce the dependency of the SKP2-CUL1 interaction on SKP1; and (*c*) the binding of human SKP1 to cyclin A has previously been shown to be bridged by SKP2 (35).

The functional consequence of the CUL1-cyclin A interaction is not known at present. One obvious relevance of the CUL1-cyclin A association is to regulate the abundance of cyclin A via the ubiquitin-dependent proteolysis pathway: (*a*) the steady-state level of the cyclin A protein oscilliates during the cell cycle, primarily owing to ubiquitin-mediated proteolysis; (*b*) both CUL1 and SKP1 have been linked to ubiquitin-mediated proteolysis. The yeast homologue of human CUL1, Cdc53p, couples a ubiquitin-conjugating enzyme, Cdc34p, to cyclins and CDK inhibitors for ubiquitination. This function of Cdc53p is mediated by its direct interaction with Skp1p (28, 29, 32, 50); and (*c*) genetic analysis of the cul-1 mutant in *C. elegans* indicated that CUL-1 has a broad function in all tissues and that loss of cul-1 function results in an accelerated $G_1$-to-S progression and hyperplasia (41). These observations suggest that CUL1 functions as a broad, negative cell cycle regulator that would be consistent with a function of CUL1 in targeting the degradation of a positive cell cycle regulator such as cyclin A. However, despite its appeal, we have failed in repeated attempts to detect an alteration in the stability or abundance of cyclin A by ecotopically expressing either wild-type CUL1, an $NH_2$-terminal fragment of CUL1, or wild-type SKP2. Therefore, it remains open that the observed CUL1-cyclin A interaction may have another role in addition to regulating the level of cyclin A protein. For example, it is not inconceivable that cyclin A may play a role in regulating the activity of CUL1 by phosphorylating CUL1 or an individual component in the CUL1 complex.

Our finding of the CUL1-cyclin A interaction raises two questions concerning the regulation of this interaction. Thus far we have detected only the CUL1-cyclin A complex in virally transformed HeLa cells. In other nontransformed cells (*e.g.,* NIH-3T3, 3T3-L1, and WI-38), there exists abundant CUL1, SKP1, and cyclin A proteins; yet, we have not been able to detect the CUL1-cyclin A complex under the conditions used for coprecipitating the CUL1-cyclin A from HeLa

*Fig. 8. In vivo* association of CUL1 and cyclin A. *A,* anti-cyclin A and anti-SKP1 immunoprecipitates derived from asynchronously growing HeLa cells were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting different portions derived from the same gel with antibodies specific to CUL1, cyclin A, and SKP1 as indicated. *B,* lysate harvested from asynchronously growing or aphidicolin-arrested HeLa cells were immunoprecipitated with anti-cyclin A antibody. Immunocomplexes were resolved by SDS-PAGE and transferred to nitrocellulose, and various portions of the same blot were analyzed by Western analysis using antibodies to CUL1 (*top*), cyclin A (*upper middle*), hSKP2 (*lower middle*), and hSKP1 (*bottom*). *C,* lysates derived from asynchronously growing HeLa cells were immunoprecipitated with antibodies specific to SKP1 and cyclins A, E (± competing peptide), D1 (± competing peptide), and B1 (or preimmune, indicated by + competing peptide). After SDS-PAGE, proteins were transferred to nitrocellulose, and various portions of the same blot were analyzed by Western analysis using antibodies to CUL1 (*top*), cyclin A (*middle*), and a mixture of CDC2 and CDK2 (*bottom*).

cells.[7] All three proteins in this cyclin A complex, CUL1, SKP1, and the F-box-containing SKP2, are now implicated in targeting specific protein substrates for ubiquitin-dependent proteolysis. Formation of CUL1/SKP1/SKP2/cyclin A complexes in HeLa cells raises the possibility that viral transformation may regulate the ubiquitin-dependent proteolytic activity associated with CUL1 whether to affect the stability of cyclin A or the function of cyclin A in regulating CUL1 activity.

The second question concerns the observation that the CUL1-SKP1 complex remains essentially unchanged during cell cycle progression. SKP1-associated CUL1 remained unaltered from S to $G_2$-M (Fig. 6), the levels of both CUL1 and SKP1 did not change during terminal cell differentiation, and both proteins were expressed at levels similar to that found in proliferating and temporarily arrested cells (Fig. 7). This finding is somewhat unexpected in light of the observation that CUL1 selectively interacts with a cyclin whose level strictly oscillates during the cell cycle and whose expression is turned off in postmitotic cells. There may be two possible explanations for this: (*a*) the CUL1-SKP1 complex may perform other yet unidentified functions rather than regulating cyclin A degradation. Association of CUL1-SKP1 may allow cyclin A to regulate CUL1 activity. Alternatively, the CUL1-SKP1 complex may not be required for the destruction of cyclin A for exit from mitosis; rather, it may be needed to prevent accumulation of cyclin A during $G_1$ and/or in postmitotic cells. A similar function has recently been proposed for the Drosophila *fizzy-related* (*FRZ*) gene, which encodes a WD-40 repeat protein (38). *FRZ* and *fizzy* (*FZY*) are both involved in down-regulation of several cyclin proteins including cyclin A, but they are functionally distinct. Whereas *FZY* is required for cell cycle progression through metaphase and for degradation of mitotic A- and B-type cyclins (51–53), *FZR* is required for preventing cyclin accumulation after mitosis for embryonic cells to exit from the cell cycle. Notably, the inability of cells to cease proliferation at the appropriate developmental stage as the result of mutation in *fzr,* but not *fzy,* is similar to the phenotype of the *C. elegans cul-1* mutant (41).

There is a striking similarity in the mechanistic detail between the human CUL1 and the yeast Cdc53p complex. Both directly interact with SKP1, and both indirectly interact with putative destruction substrate proteins through an F-box-containing protein, SKP2 in humans and Cdc4p or Grr1 in yeast. Such a high degree of evolutionary conservation suggests that this ternary complex configuration, cullin 1 (Cdc53)/SKP1/F-box protein (SCF) may be evolutionarily conserved in mammalian cells and may also function to target the proteolysis of a large number of cellular proteins. However, our finding that SKP1 selectively interacts with CUL1 but not other cullins indicates that the SCF pathway, although similarly used by the mammalian cullin 1, is not shared by other cullin members. We suggest that other cullins, if functioning similarly to CUL1 in coupling an E2 ubiquitin-conjugating enzyme to a substrate protein, may use a pathway(s) that is independent of SKP1 and perhaps F-box proteins to facilitate protein degradation.

## Materials and Methods

**Cullin, SKP1, and SKP2 Constructs.** Full-length human cullin 1 coding sequence was amplified from a HeLa cDNA library by PCR using two primers that were designed on the basis of the previously published sequence (41): a sense primer (5′-CACCATGGCGTCAACCCGGAGCCAGA-3′, an *Nco*I site incorporated for subsequent cloning is underlined), and an antisense primer (5′-AA*GGATCC*AGAAGGGTTAAGCCAAG-3′, a *Bam*HI site is underlined). This and the following PCR reactions used the following protocol: 94°C/1 min, 55°C/1 min/kb of DNA, 68°C-72°C/1 min, with a final extension of 68°C-72°C/10 min. Either Taq DNA polymerase (Life Technologies, Inc.) or Pfu polymerase (Stratagene) was used for amplification. The amplified sequence was verified by DNA sequencing and revealed that the *cul-1* cDNA amplified from HeLa cells contains a 24-amino acid insertion (SNQARGAGVPPSKSKKGQTPGGAQ) near the $NH_2$ terminus that was missing in the previously published sequence [between residues 58 and 59 in Kipreos *et al.* (41)]. This insertion, based on the presence of a similar 24-residue sequence at the same location in *C. elegans* CUL1, does not appear to be the result of PCR artifact. Amplified CUL1 was cloned directly into a TA cloning vector, pCR 2.1 vector (InVitrogen), and from there was inserted into a yeast two-hybrid vector pGTB8, a modified form of pGBT9 (54), using the *Nco*I and *Pst*I sites. This bait vector directs the expression of a fusion protein consisting of the DNA-binding domain of Gal4 (GAL4-BD, amino acids 1–147) and human CUL1. A series of CUL1 deletions was generated from both the $NH_2$- and COOH termini by digesting the full-length pGBT8-CUL1 with a restriction enzyme internal to the CUL1 coding region as follows: digestion of full-length CUL1 with *Eco*RI-generated pGBT8-CUL1(C527) that contains an in-frame fusion protein of the GAL4-BD and the COOH terminal 527-amino acid residues; digestion of full-length CUL1 with *Sal*I-generated pGBT8-CUL1(N604) that contains an in-frame fusion of GAL4-BD and the $NH_2$ terminal 604-amino acid residues; digestion of full-length CUL1 with *Hin*dIII-generated pGBT8-CUL1(N483) that contains the $NH_2$-terminal 483 residues; digestion with *Xba*I-generated pGBT8-CUL1(N326) that contains the $NH_2$-terminal 326-amino acid residues; digestion with *Eco*RI generated pGBT8-CUL1(N249) that contains the $NH_2$-terminal 249-amino acid residues; and digestion with *Cla*I-generated pGBT8-CUL1(N126) that contains the $NH_2$-terminal 126-amino acid residues. CUL1 was constructed in a pET bacterial protein expression vector (Promega Corp., Madison, WI) using the *Nco*I and *Bam*HI cloning sites.

To identify cDNA clones encoding the full-length sequences of other cullin proteins, we obtained and characterized several EST cDNA clones for each gene. The final full-length cDNA clones used in this study are as follows. Human CUL2 (AA206544), mouse CUL4A (AA271096), and human CUL5 (AA160378) were isolated by the WashU-Merck EST project and purchased from Genome System, Inc. (St. Louis, MO). Human CUL3 (AA307541) was isolated by The Institute for Genomic Research and Human Genome Science and provided by Dr. Matt Cotton at TIGR. After DNA sequencing, these four EST cDNA clones were found to contain full-length proteins except CUL 5, which misses 45 amino acids from its COOH terminus. Full-length CUL5 was obtained by PCR of a yeast two-hybrid HeLa cDNA library using the primers: 5′-ACAGAAGGTACCCTCTTC-3′, which incorporates a *Kpn*I site (underlined) and 5′-GCTCGAGCACAAACTTTACAACTTTCTGCCC-3′, which incorporates a *Xho*I site (underlined). The PCR product was digested with *Kpn*I and *Xho*I and ligated to the CUL5 EST clone (AA160378) digested with *Kpn*I and *Xho*I. The four cullin EST cDNA clones are suitable for *in vitro* translation using T7 RNA polymerase without the need of further subcloning. For expression in yeast for the two-hybrid assay, the coding sequence of each cullin gene was inserted into pGBT8, a modified form of pGBT9 (Clontech), in-frame with the GAL4 DNA binding domain. To facilitate the subcloning, a *Bam*HI and a *Xho*I site (underlined) were incorporated by PCR into the 5′ and 3′ end of human CUL2 using a sense primer (5′-GGGATCCCCATGTCTTTGAAACCAAGAGTAGTAG-3′) and an antisense primer (5′-TACTCGAGAGCGACATCACGCGACGTAGCTG-3′). For in-frame subcloning of human CUL 3 into pGBT8, *Nhe*I was introduced at the initiation ATG codon by site-directed mutagenesis (QuickChange kit; Stratagene) using the following primers (mutated mucleotides are in lowercase, and restriction sites are underlined): 5′-CCGCCGGGGAGGGGgCtAGCACCATGTCGAATC-3′. In-frame fusion of mouse cul 4A with GAL4-BD was created by ligating a *Xho*I site present in the EST cDNA cloning vector pT7T3D with the *Xho*I site in pGBT8. An in-frame fusion of human CUL5

with GAL4-BD was created by incorporating a *Bam*HI site by site-directed mutagenesis (QuickChange kit; Stratagene) using the following primers: 5′-CTTGGACTCTCGGGGGATCCCACTAGTTCTAGAGCG-3′ and 5′-CGCTCTAGAACTAGTGGGATCCCCCGAGAGTCCAAG-3′ (*Bam*HI sites are underlined) and subcloning into pGBT8 using the *Bam*HI and *Xho*I cloning sites. Full-length human cullin 1 (AF062536) and cullin 3 (AF062537) sequences have been deposited in the database.

Full-length SKP1 cDNA was obtained by rescuing a pGAD-hSKP1 prey plasmid from the yeast two-hybrid screen performed with pGBT8-CUL1 as bait. This prey vector directs the expression of a fusion protein consisting of the DNA activation domain of *Gal4* and human SKP1. Two SKP1 deletion prey constructs were generated from full-length SKP1 (AA032220) obtained from the WashU-Merck EST project and purchased from Genome System, Inc. After incorporating a *Nco*I restriction site by PCR using a primer based on the published sequence (35) (5′-AACACCATGGCTTCAATTAAG-3′), pACT2-SKP1(N91) which contains the $NH_2$-terminal 91-amino acid residues was generated by digesting full-length SKP1 with *Nco*I and *Xho*I; digestion with *Eco*RV and *Spe*I generated pBACHT-SKP1(C78), which in turn was digested with *Nco*I and *Xho*I to generate pACT2-SKP1(C78), which contains an in-frame fusion protein of *Gal4* AD with the $NH_2$-terminal 78-amino acid residues. hSKP1 was subcloned into the pET vector for bacterial protein expression (Promega) using the *Nco*I and *Bam*HI cloning sites.

Full-length SKP2 cDNA was amplified from a HeLa cDNA library by PCR using sequence-specific primers based on its sequence published previously (41): 5′-ACCATGGACGTATTTAAAACTCCCGGGCCTG-3′, which incorporates an *Nco*I site (underlined) and 5′-TACTCGAGACACCATCCTGCAATAAATAC-3′, which incorporates an *Xho*I site (underlined). Amplified SKP2 was cloned directly into a TA cloning vector, pCR 2.1 vector (InVitrogen), and from there was inserted into a yeast two-hybrid vector pACT2, using the *Nco*I and *Xho*I sites. This prey vector directs the expression of a fusion protein consisting of the activation domain of Gal4 and human SKP2.

**Bacterial Expression of CUL1 and SKP1.** pET-CUL1 and pET-SKP1 were transformed into BL21(DE3) bacteria cells. Picked colonies were grown for 4 h at 37°C with shaking and then induced with 2 μl of 0.4 M isopropyl-1-thio-β-D-galactopyranoside for 2 h. From this culture, 100 μl were centrifuged down, resuspended in 900 μl of 2× SDS-DTT loading dye, and boiled for 5 min.

**Yeast Two-Hybrid Assay.** The yeast two-hybrid assay was performed following a manufacturer's protocol (Clontech Laboratories, Palo Alto, CA) modified from Bartel *et al.* (54). A sequential transformation was conducted for the two-hybrid screen by first introducing the pGBT8-CUL1 bait plasmid into yeast strain HF7c (*MATα*, *ade2–101*, *gal4–542*, *gal80–538*, *his3–200*, *leu2–3, 112, lys2–801*, *trp1–901, ura3–52*, *URA3*::(*GAL4* 17-mers)$_3$-*CYC1-lacZ*, *LYS2*::*GAL1-HIS3*), and resultant yeast cells were used for the transformation with a HeLa cDNA library constructed in the vector pGAD-GH (55).

***In Vitro* Binding Assay.** Human CUL1, human CUL2, human CUL3, mouse CUL4A, human CUL5, and human SKP1 [$^{35}$S]methionine-labeled proteins were produced using the TNT T7 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Ten μl of the cullin proteins were individually mixed with 10 μl of SKP1 and allowed to bind on ice for 1 h. The mixtures were immunoprecipitated with anti-SKP1 antibody, separated on a denaturing polyacrylamide gel, and detected by autoradiography.

**Cell Lines, Culture Conditions, and Synchronization.** All mammalian cells were cultured in a 37°C incubator with 5% $CO_2$. HeLa (human cervix epithelioid carcinoma), WI-38 (human lung fibroblast), VA-13 (SV-40 transformed human lung fibroblast), IMR-90 (human lung fibroblast), MG63 (human osteosarcoma), NIH-3T3 (NIH Swiss mouse embryo), and 3T3-L1 (mouse embryo preadipocyte) cell lines were cultured in DMEM supplemented with 10% FBS, and CEM (human acute lymphoblastic leukemia cell line) and DG75 (human B lymphoblastoid) cells were cultured in RPMI 1640 supplemented with 10% FBS. Insect Sf9 cells were cultured in suspension in SF900 II serum-free medium (Life Technologies, Inc.) containing 0.5× penicillin/streptomycin.

Synchronization of HeLa cells was performed by treating cells with 1 μg/ml aphidicolin (Sigma Chemical Co., St. Louis, MO) for 16 h. Cells were released by thoroughly washing three times with 1× PBS and refeeding with DMEM/10% FBS before collecting at the indicated time points. For analysis by flow cytometry, $1 \times 10^6$ cells were washed in cold PBS and fixed in 100% ethanol overnight. Fixed cells were washed in 1% BSA-PBS solution and stained in the same solution containing 200 μg/ml RNase A and 50 μg/ml propidium iodide. Flow cytometry analysis of 10,000 cells was conducted using a Becton Dickinson FACScan to analyze cell size and DNA content by determining forward scatter and fluorescence intensity, respectively.

The mouse 3T3-L1 cell line (CL-173) was purchased from American Type Culture Collection (Rockville, MD) and maintained in growth medium (DMEM/10% FBS). The induction of differentiation was performed as described previously (56, 57). 3T3-L1 cells were grown to confluence, and 48 h after confluence is referred to as day 0 of the differentiation program. Density-arrested cells were induced to undergo adipogenic differentiation by switching cells from growth medium to differentiation medium [DMEM-H, 10% FBS, 10 μg/ml insulin (Life Technologies, Inc.), 0.5 mM 3-isobutyl-1-methyl-xanthine (Aldrich, Milwaukee, WI), and 1 μM dexamethasone (Aldrich)]. Forty-eight h later (day 2), the differentiation medium was withdrawn and replaced with DMEM-H containing 10% FBS and 10 μg/ml insulin. On days 4 and 6 of the differentiation program, the cells were fed with DMEM-H containing only 10% FBS. The appearance of cytoplasmic triglyceride droplets was monitored by bright-field microscopy and confirmed by staining with Oil-Red-O (58, 59). The expression of C/EBP-α, a well-characterized marker of adipocyte differentiation (57), was examined by Western blot analysis (Santa Cruz; sc-61). Control 3T3-L1 cells were grown to confluence in parallel and changed to culture medium that did not contain adipogenic inducers (DMEM/10% FBS). These cells did not accumulate cytoplasmic lipid droplets and did not express C/EBP-α.

**Antibodies and Immunochemistry Procedures.** Procedures for [$^{35}$S]methionine metabolic labeling, immunoprecipitation, immunoblotting, and several antibodies used in this study have been described previously (60). Homogenization of tissues from a B6D2 wild-type mouse was performed in NP40 buffer [containing 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitors (25 μg/ml leupeptin, 25 μg/ml aprotonin, 1 mM benzamidine, and 10 μg/ml trypsin inhibitor)] with a Polytron tissue disperser and mixer (Kinematica). Lysates were cleared by two centrifugations for 45 min each at 13,000 × *g* at 4°C. After determining protein concentration by Bradford assay (Bio-Rad protein assay; Bio-Rad, Hercules, CA), lysates containing an equal amount of protein derived from different time points during adipogenic induction, from the various cell lines, or from mouse tissues were used for immunoprecipitation and/or immunoblotting. Rabbit polyclonal anti-human CUL1 peptide antibody was raised at Pocono Rabbit Farm and Laboratories (Candeisis, PA) against the synthetic peptide CDGEKSTYSYLA, with the underlined region corresponding to amino acid residues 766–776 at the COOH terminus of human CUL1 (41). Rabbit polyclonal anti-human SKP1 peptide antibody was raised against the synthetic peptide CQVRKENQWCEEK, with the underlined region corresponding to amino acid residues 150–163 of human SKP1 (35). Both antibodies were affinity purified using respective peptide columns following the manufacturer's instruction (Sulfolink kit; Pierce, Rockford, IL). Anti-cyclin B1 antibody was raised against a fusion protein consisting of glutathione-*S*-transferase and human cyclin B1. Antibodies specific to human cyclin A (34) and cyclin D1 (61) have been described previously.

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## References

1. Reed, S. I. The role of p34 kinases in the G1 to S-phase transition. Annu. Rev. Cell Biol., *8:* 529–561, 1992.

2. Morgan, D. O. Principles of CDK regulation. Nature (Lond.), *374:* 131–134, 1995.

3. Sherr, C. J., and Roberts, J. M. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev., *9:* 1149–1163, 1995.

4. Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D., and Hunt, T. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell, *33:* 389–396, 1983.

5. Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J. Y., Hanks, S. K., Roussel, M. F., and Sherr, C. J. Identification and properties of an atypical catalytic subunit ($p34^{PSK\text{-}J3/cdk4}$) for mammalian D-type G1 cyclins. Cell, *71:* 323–334, 1992.

6. Sewing, A., Burger, C., Brusselbach, S., Schalk, C., Lucibello, F., and Muller, R. Human cyclin D1 encodes a labile nuclear protein whose synthesis is directly induced by growth factors and suppressed by cyclic AMP. J. Cell Sci., *104:* 545–554, 1993.

7. Bates, S., Parry, D., Bonetta, L., Vousden, K., Dickson, C., and Peters, G. Absence of cyclin D/cdk complexes in cells lacking functional retinoblastoma protein. Oncogene, *9:* 1633–1640, 1994.

8. Clurman, B., Sheaff, R. J., Thress, K., Groudine, M., and Roberts, J. M. Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. Genes Dev., *10:* 1979–1990, 1996.

9. Won, K-A., and Reed, S. I. Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. EMBO J., *15:* 4182–4193, 1996.

10. Parry, D., Bates, S., Mann, D. J., and Peters, G. Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of $p16^{INK4/MTS1}$ tumour suppressor gene product. EMBO J., *14:* 503–511, 1995.

11. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Sal, G. D., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science (Washington DC), *269:* 682–685, 1995.

12. Timchenko, N. A., Wilde, M., Nakanishi, M., Smith, J. R., and Darlington, G. J. CCAAT/enhancer-binding protein a (C/EBPa) inhibits cell proliferation through the p21 (WAF1/CIP-1/SDI-1) protein. Genes Dev., *10:* 804–815, 1996.

13. Maki, C. G., and Howley, P. M. Ubiquitination of p53 and p21 is differentially affected by ionizing and UV radiation. Mol. Cell. Biol., *17:* 355–363, 1997.

14. King, R. W., Deshaies, R. J., Peters, J-M., and Kirscher, M. W. How proteolysis drives the cell cycle. Science (Washington DC), *274:* 1652–1659, 1996.

15. Hershko, A. Roles of ubiquitin-mediated proteolysis in cell cycle control. Curr. Opin. Cell Biol., *9:* 788–799, 1997.

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

17. Gallant, P., and Nigg, E. A. Cyclin B2 undergoes cell cycle-dependent nuclear translocation and, when expressed as a non-destructible mutant, causes mitotic arrest in HeLa cells. J. Cell Biol., *117:* 213–224, 1992.

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

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

20. Ghiara, J. B., Richardson, H. E., Sugimoto, K., Henze, M., Lew, D. J., Wittenberg, C., and Reed, S. I. A cyclin B homolog in *S. cerevisiae*: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. Cell, *65:* 163–174, 1991.

21. Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B., and Nasmyth, K. Destruction of the CDC2/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. EMBO J., *12:* 1969–1978, 1993.

22. Yamano, H., Gannon, J., and Hunt, T. The role of proteolysis in cell cycle progression in *S. pombe*. EMBO J., *15:* 5268–5279, 1996.

23. Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Mathias, N., Kobayashi, R., Wittenberg, C., and Tyers, M. Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. Cell, *86:* 453–463, 1996.

24. Diehl, J. A., Zindy, F., and Sherr, C. J. Inhibition of cyclin D1 phosphorylation on theronine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev., *11:* 957–972, 1997.

25. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. Cyclin E-CDK2 is a regulator of $p27^{Kip1}$. Genes Dev., *11:* 1464–1478, 1997.

26. Vlach, J., Hennecke, S., and Amati, B. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. EMBO J., *16:* 5334–5344, 1997.

27. Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G., and Deshaies, R. J. Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. Science (Washington DC), *278:* 455–460, 1997.

28. Skowyra, D., Craig, K., Tyers, M., Elledge, S. J., and Harper, J. W. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell, *91:* 209–219, 1997.

29. Feldman, R. M. R., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. A complex of Cdc4p, Skp1p, and Cdc53p/Cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell, *91:* 221–230, 1997.

30. Yochem, J., and Byers, B. Structural comparison of the yeast cell division cycle gene *CDC4* and a related pseudogene. J. Mol. Biol., *195:* 233–245, 1987.

31. Goebl, M. G., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., and Byers, B. The yeast cell cycle gene *CDC34* encodes a ubiquitin-conjugating enzyme. Science (Washington DC), *241:* 1331–1335, 1988.

32. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell, *86:* 263–274, 1996.

33. Mathias, N., Johnson, S. J., Winey, M., Adams, A. E. M., Goetsch, L., Pringle, J. R., Byers, B., and Goebl, M. G. Cdc53p acts in concert with cdc4p and cdc34p to control the G1-to-S phase transition and identifies a conserved family of proteins. Mol. Cell Biol., *16:* 6634–6643, 1996.

34. Xiong, Y., Zhang, H., and Beach, D. Subunit rearrangement of cyclin-dependent kinases is associated with cellular transformation. Genes Dev., *7:* 1572–1583, 1993.

35. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. p19/Skp1 and p45/Skp2 are essential elements of the cyclin A-Cdk2 S phase kinase. Cell, *82:* 915–925, 1995.

36. Connelly, C., and Hieter, P. Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. Cell, *86:* 275–285, 1996.

37. Kominami, K., and Toda, T. Fission yeast WD-repeat protein Pop1 regulates genome ploidy through ubiquitin-proteasome-mediated degradation of the CDK inhibitor Rum1 and the S-phase initiator Cdc18. Genes Dev., *11:* 1548–1560, 1997.

38. Sigrist, S. J., and Lehner, C. F. Drosophila *fizzy-related* down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. Cell, *90:* 671–681, 1997.

39. Schwab, M., Lutum, A. S., and Seufert, W. Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. Cell, *90:* 683–693, 1997.

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

41. Kipreos, E. T., Lander, L. E., Wing, J. P., He, W-W., and Hedgecock, E. M. cul-1 is required for cell cycle exit in *C. elegans* and identifies a novel gene family. Cell, *85:* 829–839, 1996.

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

43. Yu, H., Peters, J-M., King, R. W., Page, A. M., Hieter, P., and Kirschner, M. W. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science (Washington DC), *279:* 1219–1222, 1998.

44. Fields, S., and Song, O. A novel genetic system to detect protein-protein interactions. Nature (Lond.), *340:* 245–246, 1989.

45. Stankovic, T., Byrd, P. J., Cooper, P. R., McConville, C. M., Munroe, D. J., Riley, J. H., Watts, G. D. J., Ambrose, H., McGuire, G., Smith, A. D., Sutcliffe, A., Mills, T., and Taylor, A. M. R. Construction of a transcription map around the gene for ataxia telangiectasia: identification of at least four novel genes. Genomics, *40:* 267–276, 1997.

46. Zhang, H., Xiong, Y., and Beach, D. Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol. Biol. Cell, *4:* 897–906, 1993.

47. MacDougald, O. A., and Lane, M. D. Transcriptional regulation of gene expression during adipocyte differentiation. Annu. Rev. Biochem., *64:* 345–373, 1995.

48. Smas, C. M., and Sul, H. S. Control of adipocyte differentiation. Biochem. J., *309:* 697–710, 1995.

49. Listwan, J., Marti, A., Sutterluty, H., Gstaiger, M., Wirbelauer, C., and Krek, W. Association of human CUL-1 and ubiquitin-conjugating enzyme CDC34 with the F-box protein p45SKP2: evidence for evolutionary conservation in the subunit composition of the CDC34-SCF pathway. EMBO J., *17:* 368–383, 1998.

50. Patton, E. E., Willems, A., Sa, D., Kuras, L., Thomas, D., Craig, K. L., and Tyers, M. Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and ethionine biosynthesis in yeast. Genes Dev., *12:* 692–705, 1998.

51. Dawson, I. A., Roth, S., Akam, M., and Artavanis-Tsakonas, S. Mutations of the *fizzy* locus cause metaphase arrest in *Drosophila melanogaster* embryos. Development (Camb.), *117:* 359–376, 1993.

52. Dawson, I. A., Roth, S., and Artavanis-Tsakonas, S. The Drosophila cell cycle gene *fizzy* is required for normal degradation of cyclin A and B during mitosis and has homology to the *CDC20* gene of *Saccharomyces cerevisiae*. J. Cell Biol., *129:* 725–737, 1995.

53. Sigrist, S., Jacobs, H., Stramann, R., and Lehner, C. F. Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. EMBO J., *14:* 4827–4838, 1995.

54. Bartel, P. L., Chien, C-T., Sternglanz, R., and Fields, S. Using the two hybrid system to detect protein-protein interactions. *In:* D. A. Hartley (ed.), Cellular Interactions in Development: A Practical Approach, pp. 153–179. Oxford, UK: Oxford University Press, 1993.

55. Hannon, G., Demetrick, D., and Beach, D. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev., *7:* 2378–2391, 1993.

56. Student, A. K., Hsu, R. Y., and Lane, M. D. Induction of fatty acid synthesis in differentiating 3T3–L1 preadipocytes. J. Biol. Chem., *255:* 4745–4750, 1980.

57. Yeh, W., Cao, Z., Classon, M., and McKnight, S. L. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev., *9:* 168–181, 1995.

58. Rubin, C., Hirsch, A., Fung, C., and Rosen, O. Development of hormone receptors and hormonal responsiveness *in vitro*: insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3–L1 cells. J. Biol. Chem., *253:* 7570–7578, 1978.

59. Preece, A. Manual for Histologic Technicians, p. 260. Boston, MA: Little, Brown, and Co., 1972.

60. Jenkins, C. W., and Xiong, Y. Immunoprecipitation and immunoblotting in cell cycle studies. *In:* M. Pagano (ed.), Cell Cycle: Material and Methods, pp. 250–263. New York: Springer-Verlag, 1995.

61. Xiong, Y., Zhang, H., and Beach, D. D-type cyclins associated with multiple protein kinases and the DNA replication and repair factor PCNA. Cell, *71:* 505–514, 1992.

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# ROC1, a Homolog of APC11, Represents a Family of Cullin Partners with an Associated Ubiquitin Ligase Activity

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## Summary

**We have identified two highly conserved RING finger proteins, ROC1 and ROC2, that are homologous to APC11, a subunit of the anaphase-promoting complex. ROC1 and ROC2 commonly interact with all cullins while APC11 specifically interacts with APC2, a cullin-related APC subunit. Yeast ROC1 encodes an essential gene whose reduced expression resulted in multiple, elongated buds and accumulation of Sic1p and Cln2p. ROC1 and APC11 immunocomplexes can catalyze isopeptide ligations to form polyubiquitin chains in an E1- and E2-dependent manner. ROC1 mutations completely abolished their ligase activity without noticeable changes in associated proteins. Ubiquitination of phosphorylated IκBα can be catalyzed by the ROC1 immunocomplex in vitro. Hence, combinations of ROC/APC11 and cullin proteins potentially constitute a wide variety of ubiquitin ligases.**

## Introduction

Many regulatory proteins such as cyclins, CDK inhibitors, and transcription factors are regulated by the ubiquitin-dependent proteolytic pathway (Hochstrasser, 1996; King et al., 1996; Hershko, 1997). A cascade of enzymes, E1, E2, and E3, catalyze the attachment of ubiquitin (Ub) to substrates to form polyubiquitinated conjugates that are rapidly detected and degraded by the 26S proteasome. E1 and E2 both represent structurally related and well characterized enzymes that do not provide much substrate specificity. The E3 is ambiguously defined as a function containing two separate activities: an Ub ligase activity to catalyze isopeptide bond formation and a specific targeting activity to physically bring the ligase and substrate together. Elucidating the molecular nature and the regulation of E3s have become critical issues central to our understanding of regulated proteolysis.

Knowledge about E3 Ub ligases is very limited at present. The best characterized E3 ligase is the APC (anaphase-promoting complex or cyclosome), which plays a crucial role in regulating the passage of cells through anaphase (reviewed in King et al., 1996). Most proteins known to be degraded by the APC contain a conserved 9–amino acid stretch, commonly known as the destruction box, that is necessary for their ubiquitination and degradation (Glotzer et al., 1991). Proteins that are degraded during G1 do not contain the conserved destruction box. Instead, substrate phosphorylation appears to play an important role in targeting their interaction with an E3 for ubiquitination. Genetic and biochemical analysis in yeast have identified an E3-like activity, the SCF, that plays a key role in regulating G1 progression. The SCF consists of at least three subunits, SKP1, CDC53/cullin, and an F box–containing protein, in which SKP1 functions as an adapter to connect CDC53 to the F box protein that binds directly to the substrate (reviewed in Hoyt, 1997).

Despite extensive investigations into the APC and SCF, the nature of E3 ligases still remains unclear. One inconsistency is that all in vitro reconstituted SCF ubiquitination reactions reported thus far have required the supplement of a cellular extract. This indicates that an essential protein component(s) or a critical modification was missing from the complexes assembled from isolated proteins. We report here the identification of a family of closely related RING finger proteins, ROC1, ROC2, and APC11. We present evidence that ROC1/APC11 are general cullin-binding proteins that have an associated ligase activity. Hence, ROC-cullins and APC11-APC2 may function as Ub ligases during interphase and mitosis, respectively.

## Results

### ROC1 Interacts Directly with All Cullins

In a yeast two-hybrid screen of a human HeLa pGAD-cDNA library using mouse cullin 4A as bait, we identified a gene encoding a RING finger protein, named ROC1 (regulator of cullins). ROC1 can also interact with cullins 1, 2, and 5 (Figure 1A). We then demonstrated that ROC1 interacts with the COOH-terminal 527 amino acid residues of CUL1, but not the $NH_2$-terminal 249 residues (Figure 1B). In contrast, SKP1 binds to the $NH_2$-terminal domain of CUL1. These results indicate that CUL1 contains at least two distinct domains, a $NH_2$-terminal SKP1-binding domain and a central/COOH-terminal ROC1-binding domain. Such structural separation suggests that it is unlikely that ROC1 competes with SKP1 for CUL1. Supporting this idea, we have detected ROC1 in the α-SKP1 immunocomplex, and SKP1 was reciprocally detected in the α-Roc1 immunocomplex when cells were cotransfected with ROC1, CUL1, and SKP1 (data not shown). Hence, ROC1 and SKP1 may coexist in the same protein complex with CUL1 to perform different functions.

To confirm the interaction between ROC1 and cullin proteins, Saos-2 cells were transfected with plasmids directing the expression of HA-tagged human ROC1 (HA-ROC1) together with CUL1 or other myc-epitope tagged cullins. Transfected cells were metabolically labeled with [$^{35}$S]methionine, and cell lysates were reciprocally immunoprecipitated with either α-HA, α-CUL1, or

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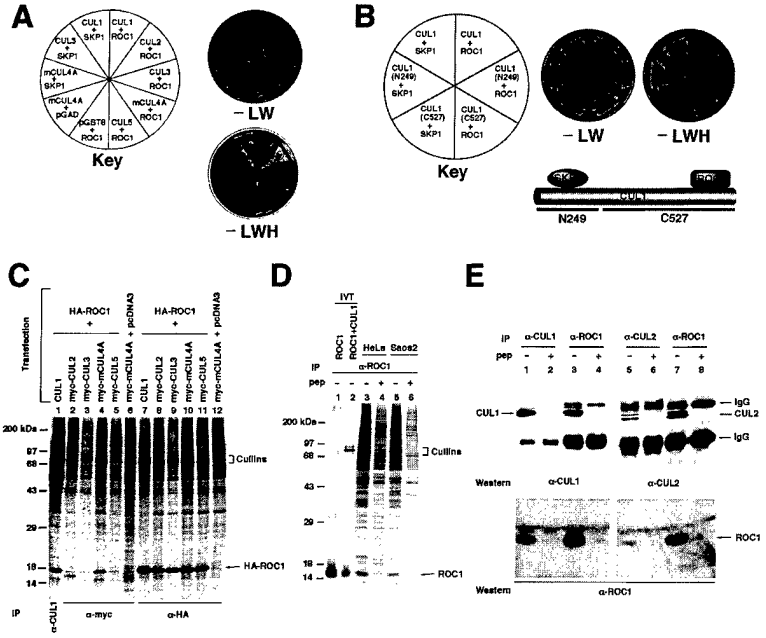


Figure 1. ROC1 Interacts with Members of the Cullin Family

(A) Yeast HF7c cells were cotransformed with plasmids expressing indicated proteins (key) and plated onto media lacking leucine and tryptophan (−LW) to verify the presence of both bait (Leu$^+$) and prey (Trp$^+$) plasmids; or onto media lacking leucine, tryptophan, and histidine (−LWH) to assay for interactions between bait and prey proteins.

(B) ROC1 interacts with the C-terminal portion of CUL1. HF7c yeast cells were cotransformed with plasmids expressing indicated proteins. Protein–protein interaction was assayed as described in (A).

(C–E) In vivo association of ROC1 with cullins.

(C) $^{35}$S-methionine-labeled lysates prepared from HeLa cells transfected with indicated plasmids.

(D) $^{35}$S-methionine-labeled, in vitro translated ROC1 and CUL1, or cell lysates from HeLa and Saos-2 cells were immunoprecipitated with indicated antibodies and resolved by SDS-PAGE.

(E) Lysates prepared from HeLa cells were immunoprecipitated with indicated antibodies with (+) or without (−) competing antigen peptide. Proteins were resolved by SDS-PAGE and analyzed by Western analysis using indicated antibodies.

α-myc antibody. All five cullins coprecipitated with ROC1 by the α-HA antibody (Figure 1C; lanes 7–11). Reciprocally, HA-ROC1 was detected in α-CUL1 and α-myc-mCUL4A immunoprecipitations (lanes 1 and 4). The association between ROC1 and cullin 1 was not disrupted by buffer containing 0.1% SDS (data not shown), indicating that the ROC1–cullins association is very stable.

To obtain evidence for in vivo ROC1–cullins association under physiological conditions, we raised rabbit polyclonal antibodies specific to ROC1. This antibody is capable of precipitating both ROC1 and the ROC1–CUL1 complex as determined by the use of in vitro translated proteins (Figure 1D; lanes 1 and 2). From metabolically labeled HeLa and Saos-2 cells, the α-Roc1 antibody precipitated a protein of approximately 14 kDa (lanes 3 and 5). This protein corresponds to ROC1 as judged by its comigration with in vitro produced ROC1 and by competition using the antigen peptide (lanes 4 and 6). Several proteins that migrated in the 90 kDa range (indicated as "cullins" in Figure 1D) were also specifically competed by the antigen peptide. We immunopurified these proteins and determined their sequences by protein microsequencing. At least four cullin proteins (CUL1, CUL2, CUL3, and CUL4A or 4B) have been identified from this analysis thus far. HeLa cell lysate was then immunoprecipitated with antibodies to ROC1, CUL1, and CUL2, and precipitates were analyzed by Western blotting. As shown in Figure 1E, both CUL1 (lane 3) and CUL2 (lane 7) were readily detected in the ROC1 immunocomplexes and were specifically competed by ROC1 antigen peptide. Reciprocally, ROC1 was detected in both CUL1 (lane 1) and CUL2 (lane 5) complexes (lower panel, Figure 1E). Thus, ROC1 is a general cullin-interacting protein.

## ROC1 Represents a Family of RING Finger Proteins Related to APC11

ROC1 encodes a 108–amino acid residue protein with a predicted molecular weight of 12265 D (Figure 2A). Database searches identified ROC1 as a highly evolutionarily conserved gene whose *S. cerevisiae* (ROC1-Sc), *S. pombe* (ROC1-Sp), and plant (ROC1-At) homologs share 67%, 88%, and remarkably 98% protein sequence identity with human ROC1, respectively, over the 82–amino acid region compared (Figure 2C). Database searches have also identified two additional genes, ROC2 in higher eukaryotes and APC11 in all eukaryotic species (Figures 2B and 2C), that are closely related to ROC1. ROC1 and ROC2 share an overall protein sequence identity of 51% with each other and 38% and 35% identity with APC11, respectively. Both ROC2 and APC11 are also highly evolutionarily conserved. Therefore, ROC1/ROC2/APC11 define a new family of proteins that are likely to carry out important cellular functions.

ROC/APC11 proteins contain two characteristic features: a RING finger and richness in tryptophan residues. The RING finger domain has been found in many eukaryotic proteins with diverse functions and is thought to mediate protein–protein interactions (Borden and Freemont, 1996). Three of the six highly conserved tryptophan residues in ROC1 are followed by an acidic amino acid residue (Asn, Glu, or Asp) that resembles the WD repeat indicating that they may be involved in mediating protein–protein interactions.

## Selective Interaction between ROC2, APC11, and Cullin Family Proteins

We next determined whether ROC2 and APC11 also interact with cullins. Almost identical to ROC1, ROC2 interacted strongly with cullins 1, 2, 4A, and 5 (Figure

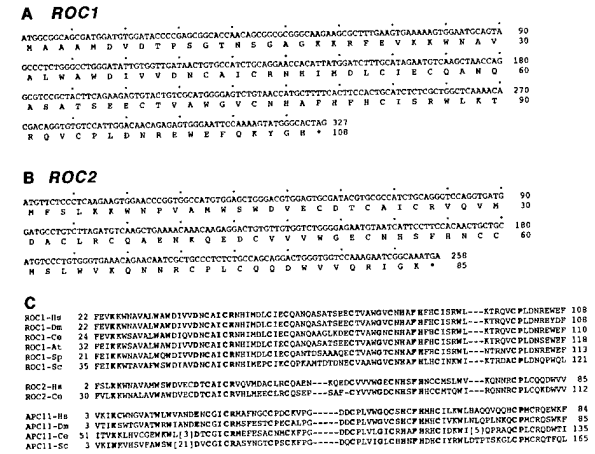


Figure 2. ROC1 Represents a Family of RING Finger Proteins Including APC11

(A) Nucleotide and amino acid sequences of human ROC1.
(B) Nucleotide and amino acid sequences of human ROC2.
(C) Sequence comparison of the ROC/APC11 family of proteins from representative organisms: human (Hs), fruit fly (Dm), nematodes (Ce), mouse ear cress (At), fission yeast (Sp), and budding yeast (Sc). Only residues that are identical to all sequences are in bold. The number in the bracket of certain sequences indicates the length of insertion omitted. The number preceding and following each sequence indicates the position of the first amino acid residue in each gene and the total length of each protein, respectively.

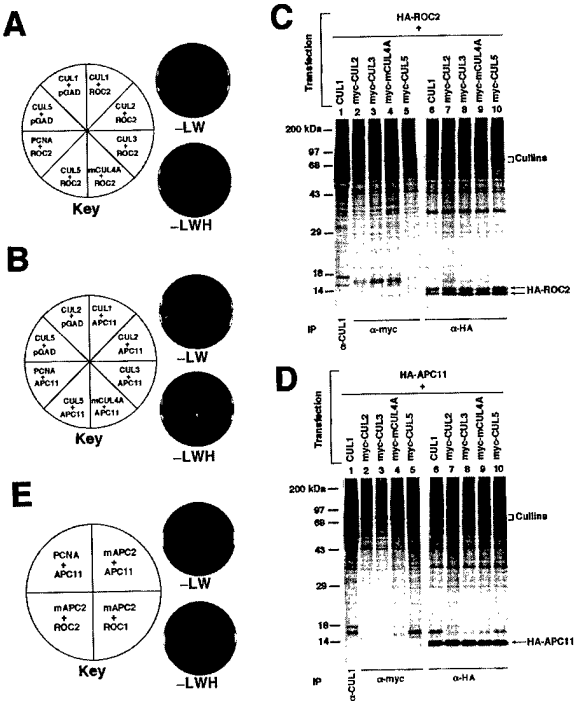


Figure 3. ROC2 and APC11 Selectively Interact with Cullins and APC2

(A and B) HF7c yeast cells were cotransformed with plasmids expressing human ROC2 or human APC11 and various cullins. pGBT8-PCNA and pGAD vector plasmid were included as negative controls. Protein–protein interactions were determined by the yeast two-hybrid assay as described in Figure 1A.
(C and D) Interaction between ROC2, APC11, and cullin family proteins in mammalian cells. Transfection followed by [$^{35}$S]IP was performed as described in Figure 1C.
(E) Selective interaction between APC2 and ROC or APC11. HF7c yeast cells were cotransformed with plasmids expressing indicated proteins (key). Protein–protein interaction was determined by the yeast two-hybrid assay using selective medium lacking histidine (−LWH) supplemented with 5 mM 3-AT to suppress the low *trans*-activating activity of GAL4$^{BD}$-APC2 fusion protein.

3A). In contrast, APC11 only interacted with cullin 5, but not other cullins (Figure 3B). Saos-2 cells were then transfected with HA-ROC2 and untagged CUL1 or myc-tagged cullins followed by [$^{35}$S]IP. All five cullins were coprecipitated with ROC2 by the HA antibody (Figure 3C; lanes 6–10). Reciprocally, ROC2 (preferentially the faster migrating form) was detected in cullin 2, 3, and 4 immunocomplexes (lanes 2–4). In contrast and with the exception of cullin 5, APC11 and cullins were not detected to interact with each other (Figure 3D; lanes 1–10). Cullin 5 was weakly but reproducibly detected in the APC11 immunocomplex (lane 10). Of all six mammalian cullins, CUL5 is the most divergent member of the cullin family and contains the highest sequence similarity to APC2. When tested by the two-hybrid assay, APC11, but not ROC1 or ROC2, interacted with mouse APC2 (Figure 3E). Consistent with this data, APC2 and APC11 were reciprocally detected in APC11 and APC2 immunocomplexes, respectively, in Saos-2 cells detected by [$^{35}$S]IP following transfection (data not shown, and see below; Figure 5B).

## Yeast Roc1p Interacts with the Yeast CDC53/Cullin Family

The yeast genome contains a single ROC gene, ScROC1 (ORF YOL133w), providing a simpler system to determine the in vivo function of ROC family proteins. We determined whether the yeast ROC/APC11 family also could directly interact with the yeast cullin/CDC53 family proteins by the yeast two-hybrid assay. The yeast genome contains four cullin members, CDC53, CUL-B (ORF YGR003w), CUL-C (ORF YJL047c), and APC2. ScROC1 interacted with all four yeast cullin genes including the most distantly related APC2. In contrast, ScAPC11 only interacted weakly with CUL-C, but not CDC53 or CUL-B (Figure 4A). Interaction of ScAPC11 with ScAPC2 could not be tested because both are self-activating as baits. Hence, like human ROC proteins, yeast ROC1 also commonly interacts with all cullins.

## Decrease of Roc1 Protein Causes a *cdc53*-, *cdc34*-, and *cdc4*-like Phenotype

We determined the consequence of deleting the ScROC1 gene by replacing it with a kanamycin resistance module by PCR homologous recombination. A 2:2 segregation was observed in 19/20 tetrads dissected on complete medium, and all of the viable colonies were kanamycin sensitive when replica plated onto selective medium. Upon microscopic inspection of the inviable spores, germination and a limited number of cell divisions to form microcolonies were observed reflecting a "maternal" supply of Roc1p (data not shown). Hence, ScROC1 is an essential gene for yeast viability.

We next created a conditional yeast strain in which

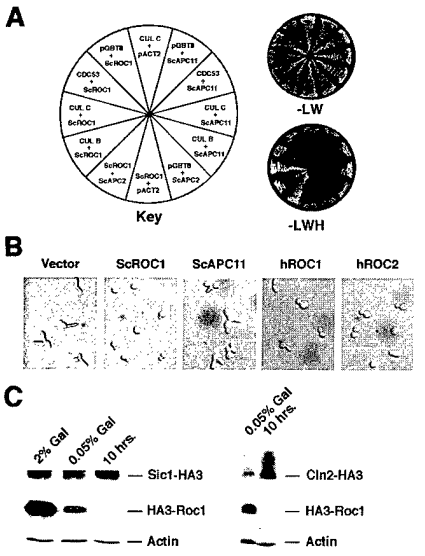


Figure 4. Function of ROC1 in Yeast

(A) ScRoc1p interacts with all yeast cullins. HF7c cells were cotransformed with plasmids expressing indicated proteins (Key). Protein–protein interactions were determined by the yeast two-hybrid assay as described in Figure 1(A).

(B) Human ROC1 and ROC2 can rescue the multibudded phenotype resulting from ScRoc1p depletion. His3MX6:P-$_{GAL}$-HA3-ROC1 haploids were transformed with pADH-414 vector, pADH-ScROC1, pADH-ScAPC11, pADH-hROC1, or pADH-hROC2. Transformants were streaked onto selective plates containing 2% glucose and grown for 24 hr. Yeasts were formaldehyde fixed and sonicated before photography.

(C) Sic1p and Cln2p accumulate in yeast depleted of ScRoc1p. Haploid His3MX6:P-$_{GAL}$-HA3-ROC1 SIC1-HA3:TRP1 and His3MX6:P-$_{GAL}$-HA3-ROC1 CLN2-HA3:TRP1 yeast were grown in either 2% or 0.05% galactose plus 2% raffinose, or 2% glucose for 10 hr. Cell lysates were resolved on an SDS-PAGE gel, transferred to nitrocellulose, and blotted with anti-HA antibody to detect Sic1p-HA3, Cln2p-HA3, and HA3-Roc1p and with anti-actin antibody to verify equal protein loading.

ScROC1 was under the control of the galactose-inducible, glucose-repressible GAL1 promoter. Repression of ScROC1 by exposure to glucose caused the yeast to begin exhibiting a mutant phenotype at 9 hr and resulted in the accumulation of a multiply elongated budded yeast population with a single nucleus by 24 hr (Figure 4B and data not shown). This Roc1p depletion–induced phenotype is indistinguishable from those caused by temperature-sensitive mutations in the CDC53, CDC4, and CDC34 genes (Mathias et al., 1996). Taking advantage of this conditional phenotype, we determined the functional conservation and specificity of ROC family proteins. The multibudded phenotype incurred by Roc1p depletion can be completely rescued by the expression of yeast ROC1, but not vector control (Figure 4B), confirming that the level of Roc1p was the rate-limiting factor causing the multibudded phenotype. Ectopic expression of both human ROC1 and ROC2 also rescued the phenotype of ScRoc1p depletion. This indicates a functional conservation of the ROC gene family. Ectopic expression of yeast APC11, however, did not rescue the phenotype (Figure 4B) demonstrating a functional specificity between members of the ROC/APC11 family.

### Roc1p Is Required for Sic1p and Cln2p Degradation

Phenotypic similarity between Roc1p depleted and cdc53 mutant cells and the interaction of ScROC1 with CDC53 prompted us to determine whether ScROC1 played a role in regulating protein degradation. Two critical substrates of the CDC53/SCF pathway are the G1 CDK inhibitor p40$^{Sic1p}$ and the G1 cyclin Cln2p (Feldman et al., 1997; Skowyra et al., 1997). As such, we asked whether Sic1p and Cln2p were stabilized in yeast depleted of ScRoc1p as determined by Western blot analysis. We created two yeast strains in which either the SIC1 gene or the CLN2 gene was HA3 epitope tagged in a GAL-HA3-ScROC1 background. Yeast cells grown in a low concentration of galactose (0.05% plus 2% raffinose) expressed a reduced level of Roc1p but still exhibited a wild-type phenotype. After 10 hr of culturing in the presence of 2% glucose to deplete ScRoc1p, Sic1p and Cln2p accumulated (Figure 4C). Protein accumulation closely correlated with the appearance of multiple elongated buds. These results provide in vivo evidence that ROC1 functions in Ub-mediated proteolysis.

### ROC1 and APC11 Complexes Contain Ub Ligase Activity

To test whether ROC1 immunocomplexes can function as Ub ligases, we immunoprecipitated ROC1 and CUL1 complexes from either 293T or HeLa cells and assayed for their ability to catalyze substrate independent Ub-Ub ligations (described in detail in Tan et al., 1999 [this issue of *Molecular Cell*]; Figure 5A). The ROC1 immunocomplex derived from both HeLa (lane 3) and 293T cells (lane 7) catalyzed the incorporation of $^{32}$P-labeled Ub into a high molecular weight smear characteristic of an incremental Ub ladder in an E1- (lane 1) and E2- (lane 2) dependent manner. Inclusion of ROC1 antigen peptide in the ROC1 immunoprecipitation effectively blocked the ligase activity (lane 4), indicating that the polyubiquitination is catalyzed by the ROC1 immunocomplex. Similarly, the CUL1 complex also exhibited the Ub ligase activity (lane 6). Substitution of E2/CDC34 with E2/UbcH5c also supported ROC1-catalyzed Ub-Ub ligation (data not shown) and Ub-substrate ligation (see below), indicating that the ROC1 complex is capable of utilizing more than one E2 enzyme.

In contrast to ROC1, the anti-APC11 complex exhibited only background levels of ligase activity when similarly incubated with E1 and E2/CDC34 (Figure 5A, lane 5). A possible explanation is that ROC1 and APC11 selectively utilizes different E2s. To test this possibility, we substituted E2/CDC34 with UbcH5c, an isoform of E2/Ubc4 (97% identity), which was previously shown to be involved in ubiquitination of mitotic cyclin B by the APC (King et al., 1995). E2-UbcH5C supported Ub ligase activity of the α-APC11 immunocomplex (Figure 5B, lane 3). α-HA immunocomplexes derived from cells transfected with HA-APC11 and myc-APC2 (lane 6), but not with empty vector and myc-APC2 (lane 5), also exhibited high levels of Ub ligase activity. The immunocomplexes associated with the ligase activity predominantly contain HA-APC11 and myc-APC2 (compare lanes 7 and 8). These results demonstrate that ROC1/APC11 proteins play a role in the activity capable of linking together two Ub molecules by an isopeptide bond to form polyubiquitin chains.

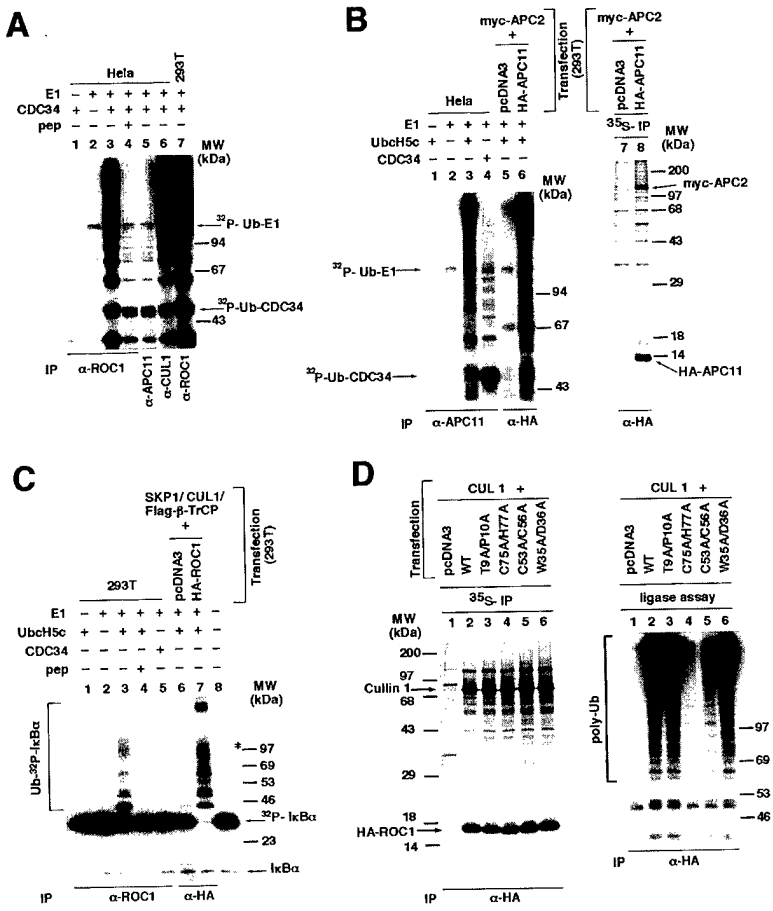


Figure 5. ROC1 and APC11 Constitute Ub Ligases with Cullin 1 and APC2

(A) Ub ligase activity of ROC1. Lysates (2 mg) from HeLa or 293T cells were immunoprecipitated with 2 μg of antibodies to either ROC1, APC11, or CUL1 as indicated. Immunocomplexes immobilized on the beads were washed and then mixed with purified E1, E2/CDC34 (unless otherwise indicated), $^{32}$P-labeled Ub, and ATP. After incubation, the reactions were terminated by boiling the sample in Laemmli loading buffer and resolved by SDS-PAGE.

(B) Ub ligase activity of APC11. Lysates from untransfected HeLa or transfected 293T cells were immunoprecipitated with indicated antibodies. Ub ligase activity was assayed with either E2/CDC34 or E2/UbcH5C as described in (A). In vivo association of transfected APC11 and APC2 was confirmed by [$^{35}$S]IP (lanes 7 and 8).

(C) In vitro ubiquitination of IκBα by ROC1 ligase. Purified IκBα was phosphorylated with IKKβ and incubated with ROC1 immunocomplexes derived from untransfected or transfected 293T cells as indicated. Lane 8 represents a total input of the phosphorylated substrate IκBα used in each reaction. The faint band (denoted by an asterisk) may correspond to the autophosphorylated form of IKKβ. After autoradiography, the gel was stained with Coomassie blue to visualize IκBα (bottom panel).

(D) Protein complex formation and ligase activity of mutant ROC1. Two sets of 293T cells were cotransfected in parallel with a CUL1 expressing plasmid with either vector control or a plasmid expressing wild-type or mutant ROC1 as indicated. Thirty-six hours after transfection, one set of cells was pulse labeled with [$^{35}$S]Met for 2 hr, lysed, and immunoprecipitated with HA antibody, and resolved by SDS-PAGE. The second set of transfected cells were lysed and immunoprecipitated with HA antibody under the same lysis and wash conditions and subjected to the Ub ligase activity assay.

Discovery of the Ub-Ub ligation catalyzed by α-Roc1 and α-APC11 immunocomplexes prompted us to determine whether they can also catalyze Ub-substrate ligations. We chose IκBα, an inhibitor of the NF-κB transcription factor, for this study, as it was recently reported that signal-induced phosphorylation of IκBα targets it for ubiquitination by cullin 1, SKP1, and the F box protein, β-TrCP (Yaron et al., 1998; Spencer et al., 1999; Winston et al., 1999). Incubation of the ROC1 complex immunoprecipitated from 293T cells with IKKβ-phosphorylated, $^{32}$P-labeled IκBα resulted in an evident accumulation of a high molecular weight species (Figure 5C). Addition of a competing ROC1 antigen peptide reduced the IκBα ubiquitination to background levels (lane 4), confirming that IκBα ubiquitination is ROC1 dependent. Ubiquitination of IκBα by ROC1 is dependent on E1 (lane 1) and E2/UbcH5C (lane 2), and substitution of UbcH5C with CDC34 failed to catalyze IκBα ubiquitination (lane 5). Compared with ROC1 complexes derived from untransfected cells (lane 3), a much higher level of ligase activity toward IκBα was obtained with HA immunocomplexes derived from HA-ROC1/CUL1/SKP1/β-TrCP transfected cells (lane 7). Note that all of the phosphorylated IκBα was converted into a high molecular weight ubiquitinated form. Coomassie blue staining of the gel after autoradiography showed that equal amounts of IκBα protein remained unubiquitinated after incubation with the ROC1 complex (Figure 5C, lane 7, bottom). This indicates that only the phosphorylated form of IκBα was ubiquitinated by the ROC1 complex.

### ROC1 Is an Essential Subunit for Ligase Activity

Demonstration of ROC1-, CUL1-, and APC11-associated Ub ligase activities led us to seek direct evidence that ROC1 is an essential subunit for ligation. We mutated several amino acid residues that are highly conserved between members of the ROC family from different species (Figure 2C). HA-tagged wild-type or mutant ROC1 was cotransfected with CUL1 into two sets of 293T cells. While one set was employed for [$^{35}$S]IP analysis, the other set was analyzed for Ub ligase activity (Figure 5D). There is no detectable difference in the ROC1-CUL1 associated proteins between wild-type and the four mutant ROC1 complexes (Figure 5D, left panel). While double mutations of T9A/P10A (Figure 5D, right panel, lane 3) and W35A/D36A (lane 6) had no detectable effect on the ligase activity of ROC1, mutation of C53A/C56A dramatically reduced ROC1-associated ligase activity (lane 5), and mutation of C75A/H77A reduced the associated ligase activity to background levels (lane 4).

These results demonstrate that ROC1 is an essential subunit for the observed ligase activity.

## Discussion

We have described an E3 ligase activity associated with a novel family of RING finger proteins, ROC/APC11, which complex with cullins to potentially constitute a large number of E3 Ub ligases. The detailed biochemical role the ROC/APC11 family plays in ubiquitination remains to be determined. Nonetheless, the presented experimental evidence supports the notion that ROC family proteins function as essential subunits of the ligase by forming heterodimers with cullins. ROC1 was shown to be an essential gene in yeast whose protein depletion caused an elongated, multibudded phenotype and concomitant accumulation of Sic1p and Cln2p (Figure 4). More convincingly, mutations in ROC1 completely abolished the associated ligase activity without a detectable alteration in the composition of ROC1-CUL1-associated proteins (Figure 5). Thus, strong evidence demonstrates that ROC1 is an essential subunit of the ligase. Though we have not definitively proven a cullin dependency for ROC1 associated ligase activity, it is likely that the cullin subunit is an obligate partner. We have demonstrated that ROC1 and ROC2 interact directly with cullins with a very high affinity, for both mammalian and yeast counterparts (Figures 1 and 4). Reinforcing this direct interaction is the in vivo association of a cullin-related protein, APC2, and a ROC protein, APC11 (Zachariae et al., 1998; Yu et al., 1998) (Figures 1 and 5B). Furthermore, in coupled transfection/ligase assays, CUL1 and ROC1 are the two predominant polypeptides in the ROC1 ligase complex. A critical issue that remains to be determined is whether the heterodimeric ROC/APC11–cullin complexes themselves contain intrinsic ubiquitin ligase activity or whether ROC/APC11 proteins act to bring the E2 and the cullins into close proximity for E2-mediated Ub-substrates ligations.

Discovery of ROC1 and APC11 as essential subunits for Ub ligation provides a clearer view of the ubiquitination pathway. In the case of the APC, identification of APC11 as a potential ligase subunit should help to determine how it is activated, inhibited, and targeted by other APC subunits. For the SCF complex, which consists of SKP1, CDC53/cullin 1, and an F box protein, it is now clear that SKP1 and the F box proteins are involved in substrate targeting. It should be pointed out that whether the SCF model can be generally applied to other cullins remains to be determined. Only cullin 1 interacts with SKP1 to be targeted by an F box protein to the substrate (Michel and Xiong, 1998), and whether higher eukaryotes contain additional SKP1-like cullin-interacting molecules is not clear. Conversely, cullin 1 and ROC1 are most likely required for ligase activity. Unlike SKP1, ROC1 and ROC2 interact commonly with all cullins that we have examined in both mammalian and yeast cells (Figures 1, 3, and 4). Hence, ROC1 and ROC2 are not just components of the SCF but essential subunits of all cullin-associated ubiquitination activities. Finally, both ROC/APC11 and cullin/APC2 represent multigene families. Among more than a dozen subunits identified, ROC/APC11 and cullin/APC2 are the only two proteins common to both the APC and the SCF complexes. The variety of combinations that the ROC/APC11 family can form with different cullins point to a potentially large number of Ub ligases, and each may be involved in a specific cellular pathway as exemplified by the function of APC11-APC2 in mitosis, ROC1-CDC53 in yeast G1 control and ROC1–cullin 1 in NF-κB/IκBα-mediated transcriptional regulation.

## Experimental Procedures

### Plasmids Constructs

Mouse cullin 4A cDNA was described (Michel and Xiong, 1998). Human ROC2 and APC11 cDNAs were isolated by PCR amplification from a HeLa cDNA library and confirmed by DNA sequencing. The mouse APC2 EST cDNA clone (W13204) was used. ROC1 mutations were introduced by site-directed mutagenesis using Quick-Change kit (Stratagene) and verified by DNA sequencing.

Yeast cDNA sequences were amplified by PCR using lyticase-treated YEF473 genomic DNA and verified by DNA sequencing. CUL B, CUL C, and ScAPC2 were PCR amplified using the long template Expand kit (Boehringer Mannheim) following manufacturer's instructions. p414-ADH vector (CEN) was used for yeast rescue experiments. pcDNA3 (Invitrogen) was used for expression in mammalian cells. pGBT8, pGAD-GH, and pACT2 vectors were used for the yeast two-hybrid experiments.

### Cell Culture

HeLa, Saos-2, and 293T were cultured in DMEM (10% FBS) in a 37°C incubator with 5% $CO_2$. Cell transfections were carried out using the LipofectAMINE reagent according to the manufacturer's instructions (GIBCO-BRL) or calcium-phosphate buffer (for 293T cells). For each transfection, 5 or 15 μg of total plasmid DNA were used for each 60 mm or 100 mm dish.

### Antibodies

Procedures for [$^{35}$S]methionine metabolic labeling, immunoprecipitation, and immunoblotting have been described previously (Jenkins and Xiong, 1995). The sequence of synthetic peptides used in generating rabbit polyclonal antibodies are as follows: anti-human ROC1N (CMAAAMDVDTPSGTN, residues 1–14), anti-human ROC1C (CDNR EWEFQKYGH, residues 97–108), anti-human APC11 (CRQEWKFKE, residues 76–84), anti-human CUL2 (CRSQASADEYSYVA, residues 733–745). Antibodies to human CUL1 and SKP1 were previously described (Michel and Xiong, 1998). Monoclonal α-HA (12CA5, Boehringer-Mannheim) and α-myc (9E10, NeoMarker) antibodies were purchased commercially. Antibody to yeast actin was provided by Dr. J. Pringle. Coupled in vitro transcription and translation reactions were performed using the TNT kit following the manufacturer's instructions (Promega).

### Yeast

All *S. cerevisiae* strains were derived from YEF473 (a/α ura3–52/ura3–52 his3Δ-200/his3Δ-200 trp1Δ-63/trp1Δ-63 leu2Δ-1/leu2Δ-1 lys2–801/lys2–801). Yeast were cultured per standard protocol (Guthrie and Fink, 1991). The procedure followed for lysing and immunoblotting has been described previously (Lamb et al., 1994). Yeast were fixed in 3.7% formaldehyde for 1 hr at 30°C, sonicated, and washed in 1× PBS.

Yeast strains were constructed using PCR-based gene deletion and modification by homologous recombination (Longtine et al., 1998). Primers for PCR products contained 40 bp of sequence homologous to the gene-specific sequence and 20 bp homologous to the vector template. PCR was performed using the Expand Long Template PCR System (Boehringer Mannheim) as described (Longtine et al., 1998). PCR products were transformed into diploid YEF473 yeast (to construct strains JM1 and JM5, see below) or into the haploid strain JM5 (to construct strain JM7 and JM8, see below)

using a standard protocol. To identify transformants that had integrated by homologous recombination, PCR was performed on genomic DNA. JM1: ROC1/roc1:kanMX6; JM5: ROC1/His3MX6:P-$_{GAL}$-HA3-ROC1; JM7: MATa His3MX6:P-$_{GAL}$-HA3-ROC1 SIC1-HA3:TRP1; JM8: MATa His3MX6:P-$_{GAL}$-HA3-ROC1 CLN2-HA3:TRP1.

### Ub Ligase Activity Assay

Human E1 was purified from HeLa cells as described (Hershko et al., 1983). Mouse E2/CDC34 was purified from insect cells infected with a mouse CDC34 expressing baculovirus, and human E2/UbcH5C were expressed in bacteria and purified using nickel beads (QIAGEN). Ub was prepared by subcloning full-length Ub as a fusion protein with a protein kinase C recognition site (LRRASV) and purified with nickel beads. Purified Ub was labeled with [$^{32}$P] by incubating with [$\gamma$-$^{32}$P]ATP and cAMP kinase (Sigma) at 37°C for 30 min. For ubiquitination assays, immunocomplexes immobilized on protein A agarose beads were washed and added to an Ub ligation reaction mixture (30 $\mu$l) that contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM NaF, 10 nM Okadaic Acid, 2 mM ATP, 0.6 mM DTT, 0.75 $\mu$g [$^{32}$P]Ub, 60 ng E1, and 300 ng E2 protein. Reactions were incubated at 37°C for 60 min. For the I$\kappa$B$\alpha$ ubiquitination assay, 8 $\mu$g of purified GST-I$\kappa$B$\alpha$ (residues 1–54) were phosphorylated with 0.05 $\mu$g of IKK$\beta$ in the presence of 4 $\mu$Ci of [$\gamma$-$^{32}$P]ATP by incubating the reaction at 30°C for 30 min in a total volume of 40 $\mu$l of kinase buffer (20 mM HEPES [pH 7.7], 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 $\mu$M ATP, 10 mM $\beta$-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 $\mu$M $Na_3Vo_4$, and 1 mM DTT). Ubiquitination of $^{32}$P-labeled GST-I$\kappa$B$\alpha$ was performed as described above, except that 1 $\mu$g of $^{32}$P-labeled GST-I$\kappa$B$\alpha$ and 12 $\mu$g of unlabeled purified bovine Ub (Sigma) were used in place of [$^{32}$P]Ub.

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### References

Borden, K.L., and Freemont, P.S. (1996). The RING finger domain: a recent example of a sequence-structure family. Curr. Opin. Struct. Biol. *6*, 395–401.

Feldman, R.M.R., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221–230.

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

Guthrie, C., and Fink, G.R., eds. (1991). Methods in Enzymology, Volume 194 (San Diego, CA: Academic Press, Inc.), pp. 273–281.

Hershko, A. (1997). Role of ubiquitin-mediated proteolysis in cell cycle control. Curr. Opin. Cell Biol. *9*, 788–799.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. *258*, 8206–8214.

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

Hoyt, M.A. (1997). Eliminating all obstacles: regulated proteolysis in the eukaryotic cell cycle. Cell *91*, 149–151.

Jenkins, C.W., and Xiong, Y. (1995). Immunoprecipitation and immunoblotting in cell cycle studies. In Cell Cycle: Materials and Methods. M. Pagano, ed. (New York: Springer-Verlag), pp. 250–263.

King, R., Peters, J., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M. (1995). A 20S complex containing cdc27 and cdc16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell *81*, 279–288.

King, R.W., Deshaies, R.J., Peters, J.-M., and Kirscher, M.W. (1996). How proteolysis drives the cell cycle. Science *274*, 1652–1659.

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

Longtine, M.S., McKenzie, A., DeMarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953–961.

Mathias, N., Johnson, S.J., Winey, M., Adams, A.E.M., Goetsch, L., Pringle, J.R., Byers, B., and Gobel, M.G. (1996). Cdc53p acts in concert with cdc4p and cdc34p to control the G1-to-S phase transition and identifies a conserved family of proteins. Mol. Cell Biol. *16*, 6634–6643.

Michel, J., and Xiong, Y. (1998). Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. Cell Growth Differ. *9*, 439–445.

Skowyra, D., Craig, K., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209–219.

Spencer, E., Jiang, J., and Chen, Z.J. (1999). Signal-induced ubiquitination of I$\kappa$B$\alpha$ by the F box protein Slimb/$\beta$-TrCP. Genes Dev. *13*, 284–294.

Tan, P., Fuchs, S.Y., Chen, A., Wu, K., Gomez, C., Ronai, Z., and Pan, Z.-Q. (1999). Recruitment of a ROC1–CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of I$\kappa$B$\alpha$. Mol. Cell *3*, this issue, 527–533.

Winston, J.T., Strack, P., Beer-Romero, P., Chu, C.Y., Elledge, S.J., and Harper, J.W. (1999). The SCF$^{\beta\text{-TRCP}}$-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in I$\kappa$B$\alpha$ and $\beta$-catenin and stimulates I$\kappa$B$\alpha$ ubiquitination in vitro. Genes Dev. *13*, 270–283.

Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A.M., Andersen, J.S., Mann, M., Mercurio, F., and Bem-Neriah, Y. (1998). Identification of the receptor component of the I$\kappa$B$\alpha$-ubiquitin ligase. Nature *396*, 590–594.

Yu, H., Peters, J.-M., King, R.W., Page, A.M., Hieter, P., and Kirschner, M.W. (1998). Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science *279*, 1219–1222.

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

### GenBank Accession Numbers

ROC1 (AF142059) and ROC2 (AF142060) sequences have been deposited into the GenBank database.

Michel, J.

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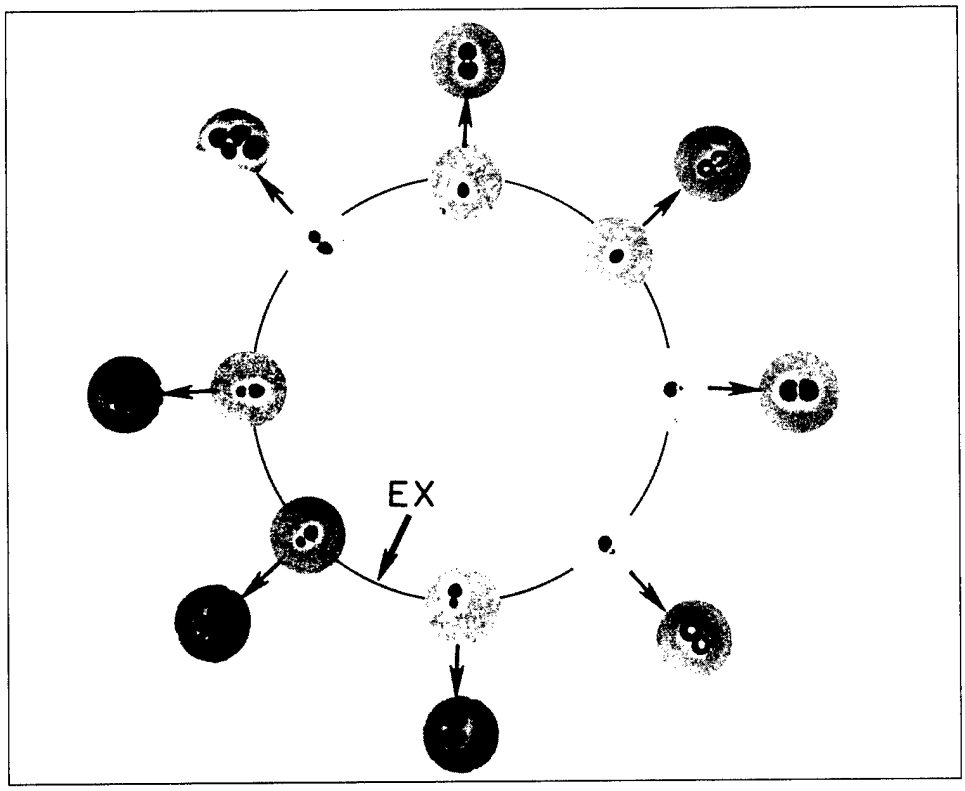


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Cold Spring Harbor, New York

## HUMAN CULLIN 1, BUT NOT OTHER CULLIN FAMILY MEMBERS, SELECTIVELY INTERACTS WITH SKP1 TO FORM A COMPLEX WITH SKP2 AND CYCLIN A

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The budding yeast gene product, CDC53p, forms E3-like SCF complexes with SKP1 and F-box containing proteins to mediate the ubiquitin-dependent degradation of G1 cyclins and CDK inhibitors. Cdc53 represents a multigene family whose human homologues, the cullin family, include at least six distinct members. We have found that human cullin 1, but not the other closely related cullins 2, 3, 4A and 5, selectively interacts with human SKP1. This CUL1-SKP1 interaction is mediated by the amino-terminal domains of both proteins, and the association appears to be required for the interaction of CUL1 with SKP2, an essential element of the S phase cyclin A-CDK2 kinase. In an asynchronous population of dividing cells, a minor amount of CUL1 specifically associates with cyclin A, but not with other cyclins or CDK inhibitors. The steady state levels of both CUL1 and SKP1 as well as their association with one another remain relatively constant throughout the cell cycle and in postmitotic cells. Our findings indicate that the SCF pathway, while similarly utilized by the mammalian cullin 1, is not shared by other cullin members. This implies that most cullins may utilize a SKP1/F-box independent pathway to facilitate protein degradation.



CONTRIBUTION OF P21
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## ...Colony Formation of Soft Tissue Sarcoma Cells is ...r Lipid-mediated Antisense Oligodeoxynucleotides ...the mdm2 Oncogene

...Mitze, Peter Würl*, Frank Bartel, Matthias Bache, ...nelore Schmidt, Friedrich-Wilhelm Rath, Helge Taubert
...ute of Pathology, University Halle-Wittenberg, D-06108 ...le/Saale, and * University Leipzig, Germany

...oft tissue sarcomas (STS) comprise a group of histologically ...terogenous and rare malignancies that are putatively mesenchymal ...origin. More than one third of human STS have elevated levels of ...e MDM2 oncogene product, resulting either from gene amplification ...alternate mechanisms. MDM2 can bind to and inhibit the tumor ...ppressor p53, and *vice versa*. Both cellular factors control ...oliferation and differentiation in normal cells and each other by fine ...ning. This balance is disrupted often in STS. The aim of this study ...as to investigate whether mdm2-antisense oligodeoxynucleotides ...S-ODN) can influence the growth characteristics of two MDM2- ...verexpressing self-established STS cell lines (US8-93, LMS6-93) ...th with heterozygous p53 non-missense mutations. Cells were ...eated with LipofectAmine™-complexed mdm2 AS-ODN (20-mer ...osphorothioates) complementary to a sequence of the mdm2 cDNA ...itiation site in comparison to sense control ODN [Kondo et al. 1995]. ...ter 24 and 48 hrs 250 cells were seeded and the clonogenic ...rvival was determined after 10 days of cultivation, respectively. ...ntrol experiments with fluorescein-labelled ODN demonstrated a ...lative highly cellular uptake of DNA (70-90%) by flowcytometry. The ...atment of US8-93 cells with AS-ODN targeted against mdm2 RNA ...t not with sense ODN control decreased the number of colonies up ...>80%. Western blot analyses demonstrated a decreasing of MDM2 ...otein level in AS-ODN transfected cells indicating an AS-specific ...hibition of mdm2 expression. We suggest that mdm2 AS-ODN ...nsfection could be an effective therapeutical strategy for STS with ...dm2 overexpression. The potential benefit of those lipid-DNA- ...mplexes in a xenograft STS perfusion model will be evaluated in ...r group.

**322** **ROC1, a homologue of APC11, represents a candidate family of cullin ubiquitin ligase regulators**

Jennifer J. Michel (1), Tomohiko Ohta (2) and Yue Xiong (1-4) (1) Curriculum in Genetics and Molecular Biology, (2) Lineberger Comprehensive Cancer Center, (3) Department of Biochemistry and Biophysics, (4) Program in Molecular Biology abnd Biotechnology University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295, USA

Cullins belong to a family of evolutionarily conserved proteins that potentially form a large number of E3-like ubiquitin ligases. We have identified two closely related novel RING finger proteins, ROC1 and ROC2, that are homologous to APC11, a subunit of the E3 APC. In order to determine its functional role, we are studying the *S. cerevisiae* ROC1 protein, whose only family members include ROC1 and APC11. Sc-ROC1 is an essential gene as determined by PCR mediated gene deletion. Depletion of Sc-ROC1 using an integrated GAL promoter system results in multi-budded yeast with one nuclei, a phenotype similar to temperature sensitive mutations in the G1/S required genes CDC53, CDC34 and CDC4. Sc-ROC1 interacts with all members of the cullin/CDC53 family: CDC53, CUL B (YGR003), and CUL C (YJL047) as well as APC2, a cullin-related component of the APC. The expression of Sc-ROC1 is relatively stable throughout the cell cycle. We are currently determining the effect of Sc-ROC1 loss on accumulation of CDC53 substrate proteins such as Cln2p and Sic1p. Research is suppported by Department of Defense Predoctoral Breast Cancer Research Fellowship (JM), in part by First Department of Surgery, St. Marianna University of School of Medicine (TO), and Public Health Service grants CA65572 and CA68377 (YX).

**323** **NUDR Contains Two DNA Contact Regions and Represses ...anscription of Heteronuclear Ribonucleoprotein A2/B1.**

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...e have recently described the cloning of human nuclear DEAF-1 related ...UDR), a novel transcriptional regulator with homology to several ...velopmental and oncogenic proteins. We have examined regions of the ...UDR protein important for DNA binding and identified heteronuclear ...onucleoprotein A2/B1 (hnRNP A2/B1) as a potential target gene. The ...mbined use of NUDR protein deletions in electrophoretic mobility shift ...says, and site-specific DNA-protein photocrosslinking techniques has ...entified two regions of NUDR capable of contacting DNA. One region ...ars resemblance to a myc-like helix-loop-helix structure and the other ...gion is proline rich and contains a Wilms' tumor-1 (WT-1) BLOCK ...gnature. The amino acid sequence between these two regions shares ...gnificant homology with the PML oncogenic domain (POD) protein SP-...0B, and our data suggests that this domain plays a highly facilitative role ...d is required for strong association with the DNA *in vitro* and ...anscriptional modulation. A database search revealed a consensus binding ...e for NUDR in the promoter of hnRNP A2/B1 and deoxyribonuclease I ...otprint assays of the promoter confirmed the presence of at least three ...nding sites for NUDR protein. In transient transfection assays, ...verexpression of NUDR reduced the activity of the hnRNP A2/B1 promoter ...r almost 60%, while NUDR constructs that were incapable of translocation ... the nucleus or that lacked either of the DNA contact regions had no effect ...n transcription. These results suggest that repression by NUDR is through a ...NA binding dependent mechanism. The inactivation of NUDR could ...ntribute to the overexpression of hnRNP A2/B1 that has been observed in ...me human cancers. (This work was supported by NIH grants HD-31613 ...d HD-32484.)

**324** **p42/p44 MAPK module plays a key role in VEGF-A transcriptionnal regulation by activating SP1 and AP-2 transcription factors**

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Vascular Endothelial Growth Factor A (VEGF-A) is a potent mitogen for vascular endothelial cells and has been implicated in tumor neovascularization. In hamster fibroblasts (CCL39 cells), VEGF-A mRNA is expressed at low levels in serum deprived or exponentially growing cells whereas it is rapidly induced after stimulation of quiescent cells with serum. To analyse the contribution of the p42/p44MAP Kinase in this induction, we used a CCL39-derived cell line (Raf-1:ER) expressing a Raf-1 chimera which can be activated by estradiol. We clearly show a time and a dose-dependent upregulation of VEGF-A mRNA detectable between 1 and 2 hrs of stimulation with estradiol. Interestingly, hypoxia has an additive effect on VEGF-A induction in CCL39 cells stimulated by serum or in Raf-1:ER cells stimulated by estradiol. We have identified a GC-rich region of the VEGF-A promoter between -88 and -66 base pairs which contains all the elements responsible of its upregulation by constitutive active Ras or MKK1. By mutation of the putative binding sites and electrophoretic mobility supershift experiments, we show that the GC-rich region constitutively binds Sp1 and AP-2 transcription factors. Furthermore, following activation of the p42/p44 MAP kinase module, the binding of Sp1 and AP-2 is increased in the complexes formed on this region of the promoter. Raf-1-initiated activation of Sp1 and AP-2 binding activity can be observed in 15 minutes, which corresponds to the peak of p42/p44 MAPK activation. *In vitro*, active p44 MAPK strongly phosphorylates Sp1 but not AP-2. We are now testing the hypothesis of a direct phosphorylation event *in vivo*.

This study demonstrates the importance of MAPK pathway in VEGF-A expression and in the mechanisms of angiogenesis.