

# Phosphorylation- and SKP1-independent *in Vitro* Ubiquitination of E2F1 by Multiple ROC-Cullin Ligases<sup>1</sup>

Tomohiko Ohta<sup>2</sup> and Yue Xiong<sup>3</sup>

Lineberger Comprehensive Cancer Center [T. O., Y. X.], Department of Biochemistry and Biophysics [Y. X.], and Program in Molecular Biology and Biotechnology [Y. X.], University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295

## Abstract

Ubiquitin-dependent proteolysis plays a critical role in the control of many cellular processes and is mediated by a cascade of enzymes involving ubiquitin activating (E1), conjugating (E2), and ligating (E3) activities. Cullin 1/CDC53 functions as an E3 ligase by interacting with RING finger protein ROC1 and recruiting phosphorylated substrate. We report here that E2F1 transcription factor can be ubiquitinated *in vitro* and *in vivo* by multiple ROC-cullin ligases. *In vitro*, E2F1 can be ubiquitinated by E2/Ubc5 but not by E2/CDC34, is dependent on catalytically active ROC1, and is protected by the Rb protein. In contrast to substrates of the SKP1-Cullin 1-F box (SCF) complexes, *in vitro* ubiquitination of E2F1 by CUL1-ROC1 ligase does not require E2F1 phosphorylation, is not stimulated by overexpression of F box protein SKP2, and is not affected by immunodepletion of SKP1 or mutations in CUL1 disrupting SKP1 binding. These results suggest a novel, SKP1-independent mechanism for targeting E2F1 ubiquitination.

## Introduction

Through a cascade of enzymes involving ubiquitin activating (E1), conjugating (E2), and ligating (E3) activities, the ubiquitin-proteasome pathway catalyzes the formation of polyubiquitin chains onto substrate proteins via isopeptide bonds. Polyubiquitinated substrates are then rapidly delivered to and degraded by the 26S proteasome (1–4). Although E1 and E2 both represent structurally related proteins and are relatively well characterized biochemically, the E3 ubiquitin ligases were conceptually defined to contain two distinct activities: a ubiquitin ligase activity that catalyzes isopeptide bond formation; and a substrate-targeting activity. One of the best characterized E3 activities is the SCF<sup>4</sup> complex in which SKP1 protein simultaneously binds to and thereby brings together CDC53/cullin 1 and an F box protein that in turn binds to a phosphorylated substrate protein (5–8). CUL1/CDC53 represents an evolutionarily conserved multigene family that includes three genes in budding yeast, seven in *Caenorhabditis elegans*, and at least six in mammalian cells (9, 10). A subunit of the mitotic APC E3 complex, APC2, was found to contain limited sequence similarity to cullins (11, 12), reinforcing the notion that cullins function in proteolysis.

Received 9/18/00; accepted 12/28/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This study was supported by Research Project Grant RPG-00-048 from the American Cancer Society (to Y. X.). T. O. is supported in part by the Department of Surgery, St. Marianna University School of Medicine, Japan. Y. X. is the recipient of a Career Development Award from the Breast Cancer Research Program of the United States Army Medical Research and Materiel Command and is a Pew Scholar in Biomedical Science.

<sup>2</sup> Present address: Department of Surgery, St. Marianna University School of Medicine, Kawasaki 216, Japan.

<sup>3</sup> To whom requests for reprints should be addressed, at Lineberger Comprehensive Cancer Center, CB 7295, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295. Phone: (919) 962-2142; Fax: (919) 966-8799; E-mail: yxiong@email.unc.edu.

<sup>4</sup> The abbreviations used are: SCF, SKP1-Cullin 1-F box; APC, anaphase-promoting complex; ROC, regulator of cullins; CDK, cyclin-dependent kinase; IP, immunoprecipitation; LLnL, *N*-acetyl-leuciny-l-leuciny-norleucinal; HA, hemagglutinin antigen.

Previously, we and others identified a RING finger protein known as ROC1 (13, 14), also called Rbx1 for RING-box protein (15, 16) or Hrt1 (17), as an essential subunit of CUL1/CDC53 ubiquitin ligases in catalyzing F box-dependent ubiquitination of phosphorylated IκBα, G1 cyclin Cln2, and CDK inhibitor Sic1. Deficiency of yeast *ROC1/Rbx1/Hrt1* can be functionally rescued by mammalian *ROC1* and its homologue *ROC2* but not yeast *APC11*, which shares a high degree of sequence similarity with ROC1 (13, 15, 17), demonstrating an evolutionary conservation and functional specificity for the *ROC* gene family (18–20). ROC1 and ROC2 commonly interact with all cullins (13), suggesting the existence of a potentially large number of heterodimeric ROC-cullin complexes. *In vivo*, there exists a large number of RING finger proteins. Several RING finger proteins with diverse structure and function, including oncoprotein MDM2 (21), tyrosine kinase negative regulator c-Cbl (22), and several poorly uncharacterized RING finger proteins (23), were linked to ubiquitination, suggesting a broad and general function of RING fingers in activating E3 ligase activity. Surprisingly, APC11 alone, in the absence of cullin-like APC2, can interact with UBC4 and is sufficient to promote E1- and E2-dependent multiubiquitin chain formation (24, 25). These findings are consistent with an idea that cullins function as scaffold proteins to bring together the RING-E2 ligase and substrates, as opposed to participation in the catalysis directly.

The *E2F* family of transcription factors controls the expression of several genes involved in the G<sub>1</sub>-to-S transition and in DNA replication. Ectopic overexpression of the prototypic member, *E2F1*, can induce quiescent cells to enter S-phase, followed by apoptosis, and can elicit neoplastic transformation in immortalized rodent cells (26, 27). Conversely, mice deficient for *E2F1* exhibited impaired apoptosis and increased tumor incidence (28, 29). These results indicate the importance of proper control of the intracellular concentration of E2F1, which is tightly regulated during the cell cycle by both transcriptional activation and ubiquitin-dependent degradation (26, 27). Although transcriptional activation of the *E2F1* promoter by one or more E2F species during G<sub>0</sub> exit has been identified as the mechanism largely responsible for the accumulation of *E2F1* mRNA in late G<sub>1</sub>, the mechanism underlying ubiquitin-mediated E2F1 degradation during late S is unclear. The only regulatory signal that has been clearly linked to the control of E2F1 stability is the binding with, and protection from, degradation by the retinoblastoma protein, both *in vitro* (30–32) and *in vivo* (33). In this report, we demonstrate that multiple ROC-cullin ligases can specifically catalyze E2F1 ubiquitination *in vitro* through a SKP1- and substrate phosphorylation-independent manner.

## Materials and Methods

**Plasmids and Purification of Recombinant Proteins.** Full-length mammalian *cullin*, *ROC1*, *ROC2*, *APC11*, *APC2*, *SKP1*, and *SKP2* expression plasmids were described by Ohta *et al.* (13) and Michel and Xiong (34). The β-TrCP clone was a gift from Dr. Yinon Ben-Neriah (The Hebrew University-Hadassah Medical School, Jerusalem, Israel). *E2 Ubc5c* was amplified from a

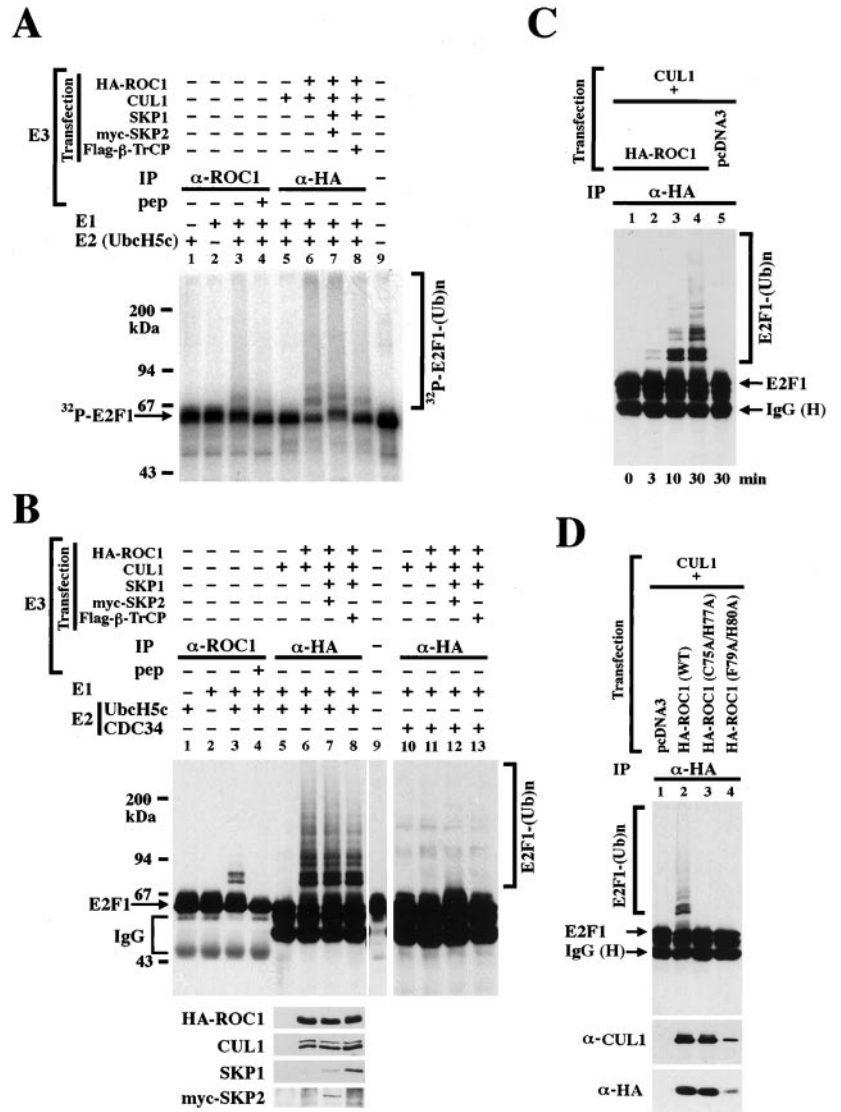


Fig. 1. *In vitro* ubiquitination of E2F1 by ROC1-CUL1. **A**, purified E2F1 was phosphorylated and <sup>32</sup>P-labeled with CDK2-cyclin A and incubated with E1, E2, ubiquitin, and ROC1 immunocomplexes derived from untransfected or transfected 293T cells as indicated. **B**, *in vitro* E2F1 ubiquitination was performed as in **A**, except that unphosphorylated E2F1 was used as a substrate and E2F1 ubiquitination was examined by anti-E2F1 immunoblotting. Association of CUL1, SKP1, and myc-SKP2 with HA-ROC1 was confirmed by immunoblotting the HA-ROC1 immunoprecipitate with antibodies to HA, CUL1, SKP1, and myc, respectively (*bottom panel*). **C**, purified recombinant E2F1 was incubated with HA-ROC1/CUL1 immunocomplexes for various lengths of time, and E2F1 ubiquitination was examined by anti-E2F1 immunoblotting. **D**, 293T cells were cotransfected with cullin 1 and either vector DNA control, wild type, or two ROC1 mutants. ROC1-CUL1 complex formation was examined by coupled IP-Western blot (*bottom panel*), and ubiquitination of E2F1 (unphosphorylated) was examined by anti-E2F1 immunoblotting.

HeLa cDNA library by PCR and inserted into a T7 bacterial expression vector fused in-frame with a hexahistidine tag. A GST-Rb<sup>379-928</sup>-expressing plasmid was a gift from Dr. Jiri Lukas (Danish Cancer Society, Copenhagen, Denmark), and an HA-Ub-expressing plasmid was a gift from Dr. Dirk Bohmann (EMBL, Heidelberg, Germany). Purified rabbit E1 (Exeter, United Kingdom) and ubiquitin (Sigma) were purchased commercially. Ubc5c, E2F1, and p21 proteins were expressed in bacteria using the pET-3E-6xHis vector with isopropyl-1-thio- $\beta$ -D-galactopyranoside induction, purified using nickel beads (Qiagen) according to the manufacturer's instructions, and stored with 10% glycerol at -80°C. Hexahistidine-tagged mCDC34 was expressed using a baculovirus and purified from Sf9 insect cells. GST-Rb<sup>379-928</sup> fusion protein was expressed in bacteria overnight at 25°C with isopropyl-1-thio- $\beta$ -D-galactopyranoside and purified with glutathione agarose beads according to the manufacturer's instructions (Sigma). The concentrations of all of the purified proteins were determined by Coomassie Brilliant Blue staining.

**Cell Culture and Immunological Techniques.** 293T cells were cultured in DMEM, supplemented with 10% FBS in a 37°C incubator with 5% CO<sub>2</sub>. Cell transfections were carried out using calcium-phosphate buffer. For each transfection, 15 or 45  $\mu$ g of total plasmid DNA were used for a 100- or 150-mm dish, respectively. Procedures for immunoprecipitation and immunoblotting have been described previously (35) with modification of the lysis buffer [15 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.35% NP40, 1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml trypsin inhibitor, and 150  $\mu$ g/ml benzamide]. For immunodepletion, all steps were carried out at 4°C. One hundred  $\mu$ l of protein A beads were incubated

with either 1 ml of anti-HA supernatant, anti-myc supernatant, or SKP1 sera for 1 h. Approximately 1.5 mg of cell lysate from HA-ROC1/CUL1-transfected cells were incubated with 30  $\mu$ l of each antibody-coated beads for three time periods (3 h, 3 h, and overnight) and once with 30  $\mu$ l of uncoated protein A beads for 1 h to remove residual antibody. Ten  $\mu$ l of the lysate after depletion were subjected to direct Western, whereas 200  $\mu$ l of the lysate were used for immunoprecipitation (with anti-HA antibody) and E2F1 ubiquitination assay. Rabbit polyclonal anti-SKP1 and anti-cullin 1 antibodies (34) and ROC1 antibody (13) were characterized previously. Anti-E2F1 antibody (clone SQ41; NeoMarkers) was purchased commercially.

**E2F1 Kinase Assay.** For E2F1 phosphorylation, active CDK2-cyclin A kinase was immunoprecipitated from 1 mg of lysate from Sf9 insect cells coinfecting with CDK2- and cyclin A-expressing baculoviruses using 3  $\mu$ l of rabbit anti-CDK2 serum. The CDK2 immunocomplexes immobilized on protein A agarose beads were washed three times with NP40 (0.5%) lysis buffer, twice with the kinase buffer [50 mM HEPES (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.4 mM Na<sub>3</sub>VO<sub>4</sub>, and 0.4 mM NaF], and added to 100  $\mu$ l of kinase reaction mixture containing 10  $\mu$ g of 6xHis-E2F1 and 50  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. Reactions were incubated at 30°C for 30 min, and 5  $\mu$ l of the supernatant were subjected to either SDS-PAGE or the ubiquitination assay. For the p21 kinase inhibition assay, 3  $\mu$ g of purified 6xHis-p21 were added to 1 mg of the cell lysate prior to immunoprecipitation.

**Ubiquitin Ligase Activity Assay.** Different ROC and cullin immunocomplexes were precipitated from either untransfected 293T cells with affinity-purified anti-ROC1 (1.5  $\mu$ g) antibody or from transfected cells with 3  $\mu$ g of

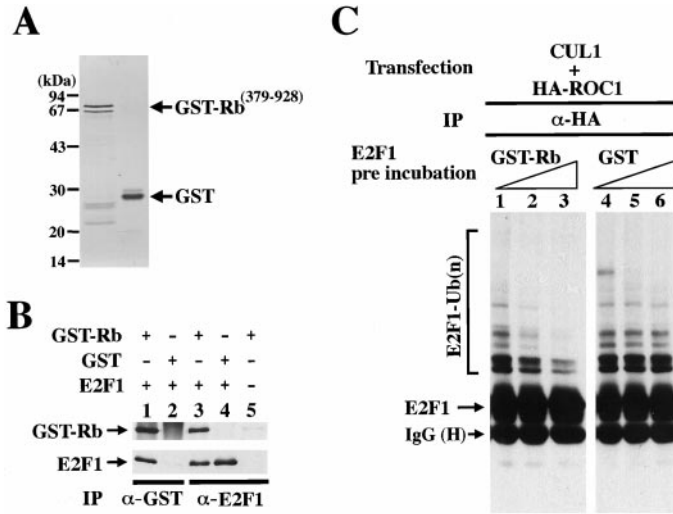


Fig. 2. Rb protein prevents *in vitro* E2F1 ubiquitination. **A**, purified GST-Rb<sup>379-928</sup> and GST proteins were examined by Coomassie Blue staining. **B**, 0.9  $\mu$ g of purified GST-Rb<sup>379-928</sup> or GST was mixed with 0.5  $\mu$ g of purified E2F1 protein at 4°C overnight. Mixtures were precipitated with anti-GST or anti-E2F1 antibody as indicated, and immunoprecipitates were resolved by SDS-PAGE. Rb-E2F1 complex formation was examined by anti-GST and anti-E2F1 immunoblotting. **C**, 0.3 (Lanes 1 and 4), 0.6 (Lanes 2 and 5), and 0.9  $\mu$ g (Lanes 3 and 6) of GST-Rb or GST protein were first incubated with 0.5  $\mu$ g of E2F1 protein at 4°C overnight before ubiquitination assay.

affinity purified anti-CUL1, anti-HA, or anti-myc antibody. Individual immunocomplexes were immobilized on protein A agarose beads, washed three times with lysis buffer, and washed twice with a buffer containing 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.01% NP40, and 10% glycerol. Washed immunocomplexes were added to a ubiquitin ligation reaction (final volume, 30  $\mu$ l) containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 2 mM NaF, 10 nM okadaic acid, 2 mM ATP, 0.6 mM DTT, 60 ng of E1, 300 ng of E2, 0.5  $\mu$ g of purified His-E2F1, and 12  $\mu$ g of unlabeled purified bovine ubiquitin (Sigma). Reactions were incubated at 37°C for 30 min unless otherwise indicated, terminated by boiling for 5 min with SDS-sample buffer containing 0.1 M DTT, and resolved by SDS-PAGE, followed by immunoblotting with an anti-E2F1 antibody. Ubiquitination of phosphorylated E2F1 was performed the same as described above using [<sup>32</sup>P]His-E2F1 phosphorylated by cyclin A-CDK2 enzyme. For Rb protection of E2F1 ubiquitination, 0.5  $\mu$ g of purified His-E2F1 was first incubated with the indicated amounts of either GST or GST-Rb<sup>379-928</sup> in PBS buffer in a total volume of 15  $\mu$ l at 4°C for overnight. Mixture was then subjected to either IP-Western to confirm Rb-E2F1 complex formation or to the ubiquitin ligase assay.

For *in vivo* E2F1 ubiquitination assay, 293T cells on a 100-mm dish were transfected with appropriate plasmids expressing HA-Ub (2.5  $\mu$ g), E2F1 (2.5  $\mu$ g), CUL1 (10  $\mu$ g), and CUL3 (10  $\mu$ g). The total amount of plasmid DNA in each transfection was adjusted to a final 15  $\mu$ g with pcDNA3 empty vector when needed. Thirty-six h after transfection, cells were treated with proteasome inhibitor LLnL (50  $\mu$ M) for 4 h. Cells were then collected, pelleted by centrifugation, lysed in 200  $\mu$ l of preboiled lysis buffer [50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1% SDS, and 1 mM DTT], and further boiled for an additional 10 min. Lysates were clarified by centrifugation at 14,000 rpm on a microcentrifuge for 10 min. Supernatant was diluted 10 times with 0.5% NP40 buffer and immunoprecipitated with anti-E2F1 antibody (3  $\mu$ g). Immunoprecipitates were washed three times and resolved by 7.5% SDS-PAGE, followed by immunoblotting with anti-HA antibody (1  $\mu$ g/ml).

## Results

**In Vitro Ubiquitination of E2F1 by ROC1-CUL1 Ligase.** Isolation of ROC1 and development of an *in vitro* ubiquitination assay of ROC1-CUL1 ubiquitin ligase purified by affinity immunoprecipitation allowed us to begin examining *in vitro* ubiquitination of specific substrates. By using this coupled immunoprecipitation and *in vitro* ubiquitination reaction assay, we have tested ubiquitination of several candidate substrates that are known to be ubiquitinated

*in vivo*. I $\kappa$ B $\alpha$ , an inhibitor of the transcription factor NF- $\kappa$ B, and E2F1, a member of the E2F transcription factor family, were found to be efficiently ubiquitinated *in vitro* by the ROC1-cullin immunocomplexes. SCF <sup>$\beta$ -TrCP</sup>-dependent ubiquitination of phosphorylated I $\kappa$ B $\alpha$  by ROC1-CUL1 ligase has been reported elsewhere (13), and characterization of E2F1 *in vitro* ubiquitination is presented in this report. Recombinant human E2F1 protein was expressed and purified from bacteria and phosphorylated with CDK2-cyclin A in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. A faint high molecular weight smear was detected when the phosphorylated E2F1 was incubated with the anti-ROC1 complex immunoprecipitated from untransfected 293T cells (Fig. 1A, Lane 3). The <sup>32</sup>P smear became more obvious when incubated with the HA immunocomplex from 293T cells overexpressing HA-ROC1 and cullin 1 (Fig. 1A, Lane 6). Such a smear was not seen when either E1 (Fig. 1A, Lane 1) or E2 (Fig. 1A, Lane 2) was omitted, when a molar excess of competing antigen peptide was added to ROC1 immunoprecipitation (Fig. 1A, Lane 4), or when the HA immunoprecipitate was derived from cells transfected with cullin 1 alone (Fig. 1A, Lane 5).

To confirm that the high molecular weight <sup>32</sup>P smear corresponded to ubiquitinated E2F1 and to determine whether CDK2-cyclin A phosphorylation is required for E2F1 ubiquitination, a similar *in vitro* ubiquitination assay was performed using unphosphorylated E2F1 protein, followed by detection with anti-E2F1 immunoblotting. The pattern of E2F1 ubiquitination in this series of experiments was almost the same as that of the phosphorylated, <sup>32</sup>P-labeled E2F1. Two slow-migrating E2F1 species were detected when incubated with ROC1 immunocomplexes from untransfected cells (Fig. 1B, Lane 3) in an E1 (Fig. 1B, Lane 1)- and E2/Ubc5c (Fig. 1B, Lane 2)-dependent manner, and the addition of competing ROC1 antigen peptide (Fig. 1B, Lane 4) abolished E2F1 ubiquitination. A significantly higher amount of E2F1 ubiquitination was detected when the ROC1-CUL1 ligase was derived from cells overexpressing HA-ROC1 and cullin 1 (Fig. 1B, Lane 6), suggesting that ROC1 and/or cullin 1 is a rate-limiting factor(s) for E2F1 ubiquitination in this assay. Incubation with HA immunocomplexes from cells transfected with cullin 1 alone did not result in E2F1 ubiquitination (Fig. 1B, Lane 5), excluding the possibility of nonspecific precipitation of E2F1 ligase activity by the HA antibody. ROC1-CUL1-catalyzed E2F1 ubiquitination requires Ubc5c and cannot use CDC34 (Fig. 1B, Lanes 10-13), indicating an E2-substrate selectivity. E2F1 polyubiquitination is catalyzed by ROC1-CUL1 in a time course-dependent manner (Fig. 1C). E2F1 was not ubiquitinated by the ROC1<sup>F79A/H80A</sup> mutant that impairs the association of ROC1 with cullin 1 (34) or by the ROC1<sup>C75A/H77A</sup> mutant that inactivates ROC1-CUL1 ligase activity without disrupting their association (13), further confirming that E2F1 ubiquitination is catalyzed by the ROC1-CUL1 ligase (Fig. 1D). Ubiquitination of recombinant, unphosphorylated E2F1 also suggests that *in vitro* E2F1 ubiquitination by ROC1-CUL1, unlike substrates of the CUL1-dependent SCF, may not require substrate phosphorylation (see below).

Recently, p45<sup>SKP2</sup> was implicated in targeting E2F1 ubiquitination (36). The function of SKP2 has also been linked to the ubiquitination of phosphorylated CDK inhibitor p27 (37-39). We determined whether SKP2 overexpression enhanced E2F1 ligase activity. Whether phosphorylated (Fig. 1A) or unphosphorylated (Fig. 1B) E2F1 was used, cotransfection of SKP1 (Lanes 7 and 8), p45<sup>SKP2</sup> (Lane 7), or another F box protein,  $\beta$ -TrCP (Lane 8) did not detectably increase the E2F1 ligase activity of ROC1-CUL1. Both SKP1 and SKP2 were detected in anti-HA-ROC1 immunocomplexes (Fig. 1B, Lanes 7 and 8, bottom panel), excluding the possibility that the inability of SKP2 and SKP1 to enhance E2F1 ligase activity by ROC1-CUL1 is attributable to a failure in complex assembly. Given

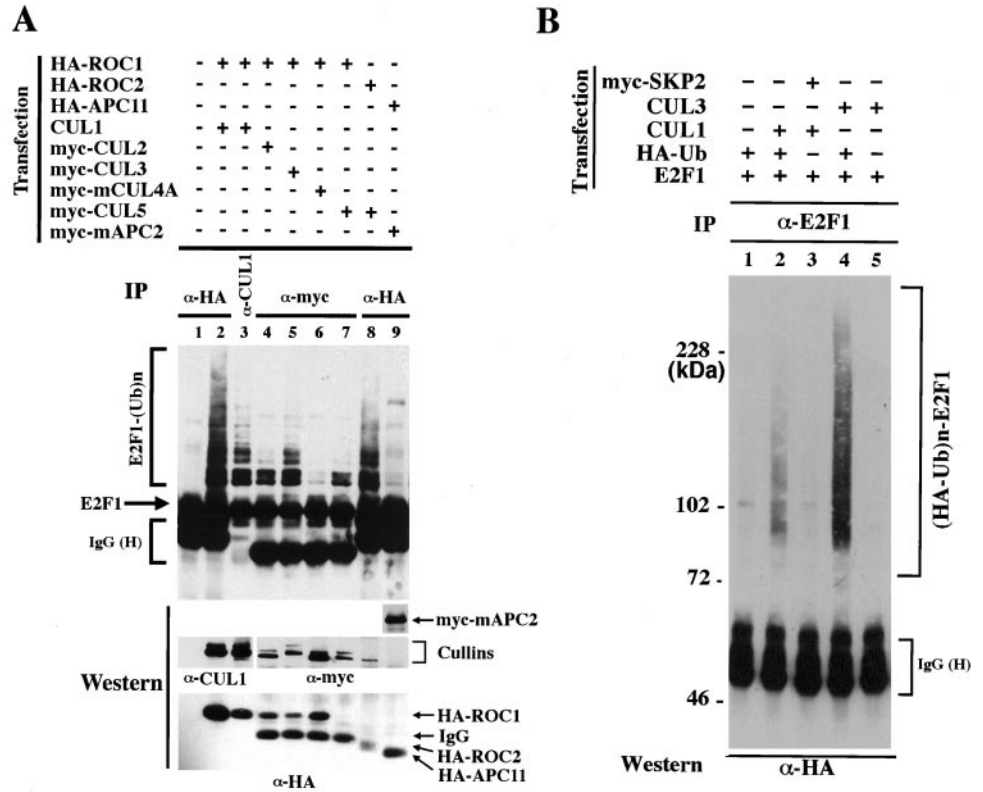


Fig. 3. *In vitro* and *in vivo* ubiquitination of E2F1 by multiple ROC-cullin ligases. *A*, 293T cells were transfected with indicated plasmids. Individual ROC-cullin ligase complexes were precipitated with either anti-CUL1, anti-HA, or anti-myc antibody and incubated with purified (unphosphorylated) E2F1 in the presence of E1, Ubc5, and ubiquitin. The reaction mixture was resolved by SDS-PAGE before E2F1 immunoblotting. Expression of transfected ROCs and cullins and ROC-cullin complex assembly was determined by IP-Western blot (*bottom panel*). *B*, 293T cells were transfected with indicated plasmids. Thirty-six h after transfection, cells were treated with proteasome inhibitor LLnL (50  $\mu$ M) for 4 h before cell lysis. Clarified cell lysate was immunoprecipitated with anti-E2F1 antibody, and washed immunoprecipitates were resolved by SDS-PAGE before immunoblotting with anti-HA antibody.

that overexpression of ROC1 and cullin 1 significantly enhanced E2F1 *in vitro* ubiquitination (Fig. 1*B*, Lane 6) and that E2F1 is in excess, these observations suggest that SKP2 (or  $\beta$ -TrCP) and SKP1 are not rate-limiting factors for E2F1 ubiquitin ligase activity of ROC1-CUL1 under these experimental conditions.

**Retinoblastoma Gene Product Protects E2F1 from Ubiquitination *in Vitro*.** The retinoblastoma gene product, Rb, binds to and protects E2F1 from degradation by the ubiquitin-proteasome pathway *in vivo* (30–33). To confirm further the specificity of *in vitro* E2F1 ubiquitination by the ROC1-CUL1 ligase, we tested whether Rb protein protects E2F1 ubiquitination *in vitro*. We purified from bacteria a GST-Rb<sup>379–928</sup> fusion protein (Fig. 2*A*) and confirmed its binding with E2F1 *in vitro* (Fig. 2*B*). When preincubated with E2F1, purified GST-Rb<sup>379–928</sup>, but not GST control, efficiently blocked *in vitro* E2F1 ubiquitination by ROC1-CUL1 in a dose-dependent manner (Fig. 2*C*). These results underscore the specificity of *in vitro* ubiquitination of E2F1 by the ROC1-CUL1 ligase and provide the first *in vitro* evidence that Rb indeed protects E2F1 from ubiquitination.

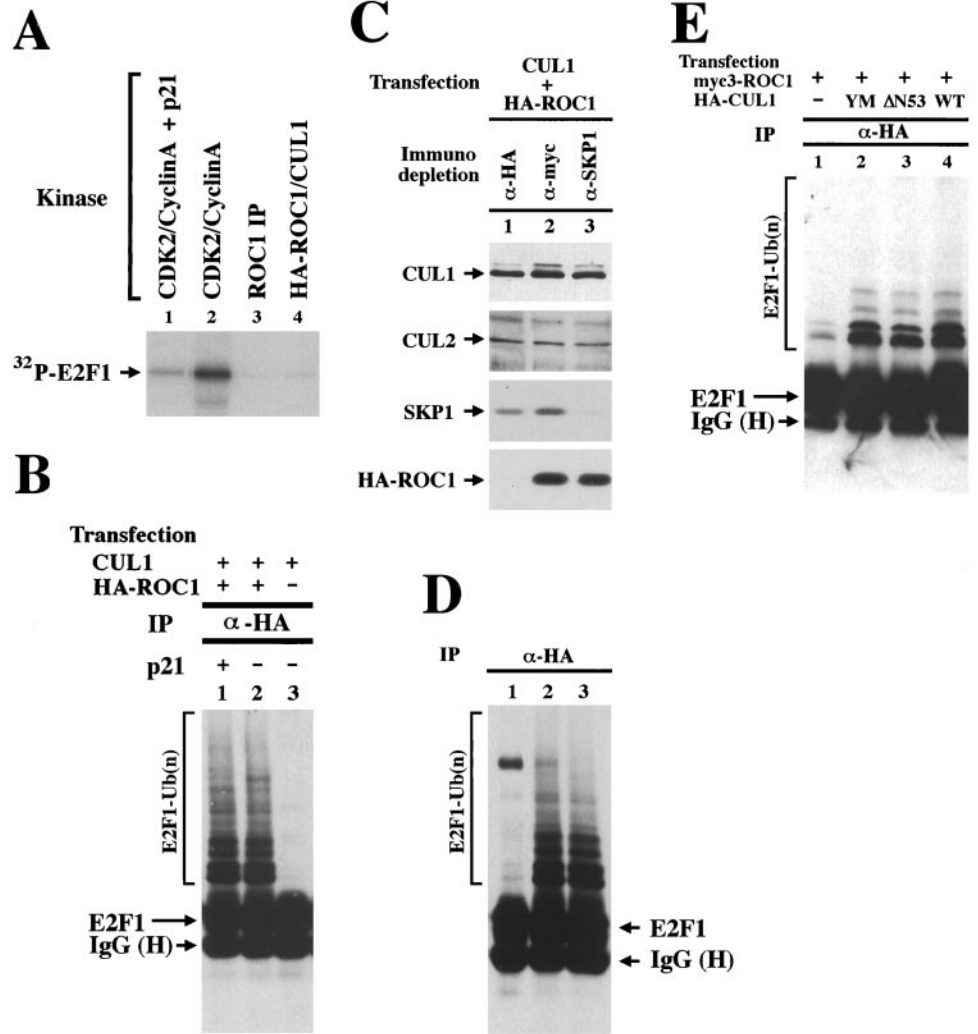
**E2F1 Can Be Ubiquitinated by Multiple ROC-Cullin Ligases.** Cullin 1/*CDC53* represent a multigene family, containing three distinct genes in yeast and at least six in mammalian cells [cullins 1, 2, 3, 4A, 4B, and 5 (9)]. Different cullins commonly interact with both ROC1 and ROC2 (13). These findings suggest the possibility that other cullins and ROC2, similar to CUL1 and ROC1, may also function as ubiquitin ligases. We demonstrated recently that all five cullins that we have examined, including CUL1, CUL2, CUL3, CUL4A, and CUL5, constitute active ubiquitin ligases with ROC1 and ROC2, as determined by the formation of polyubiquitin chains in the absence of a substrate.<sup>5</sup> The ability to assay individual cullin and ROC-associated ubiquitin ligase activity led us to determine whether other ROC-cullin ligases can also catalyze E2F1 ubiquitination. Pu-

rified recombinant E2F1 (unphosphorylated) was incubated with CUL1 or myc immunocomplexes derived from cells transfected with HA-ROC and individual myc-tagged cullins. Six of eight immunocomplexes tested, HA-ROC1 (Fig. 3*A*, Lane 2), CUL1 (Lane 3), myc-CUL2 (Lane 4), myc-CUL3 (Lane 5), myc-CUL 5 (Lane 7), and ROC2 (Lane 8), were capable of catalyzing E2F1 ubiquitination with varying degrees of efficiency in the presence of E1 and E2/Ubc5. E2F1 ubiquitination was not detected in myc-CUL4A or HA-APC11 immunocomplexes. An inability to catalyze E2F1 ubiquitination by these two ligases is not attributable to a low level of expression (Fig. 3*A*, *bottom panel*), the lack of intrinsic activity (confirmatory data not shown), or a failure in complex assembly (Fig. 3*A*, *bottom panel*). These results suggest substrate specificity of individual ROC-cullin ligases. Further supporting the E3-substrate specificity and consistent with its documented SCF-dependent ubiquitination, only ROC1-CUL1, but not other ROC-cullin ligases, is capable of catalyzing the ubiquitination of phosphorylated I $\kappa$ B $\alpha$  (data not shown). The myc-CUL5 immunocomplex derived from myc-CUL5 and HA-ROC1 cotransfected cells, although displaying E2F1 ligase activity (Fig. 3*A*, Lane 7), does not contain detectable HA-ROC1 (*bottom panel*). This observation suggests that CUL5 may prefer ROC2 as its ROC partner.

*In vitro* ubiquitination of E2F1 by ROC-cullin ligases led us to determine whether cullins can also stimulate E2F1 ubiquitination *in vivo*. 293T cells were cotransfected with plasmids expressing HA-tagged ubiquitin (HA-Ub), E2F1, and different cullins. Transfected cells were treated with proteasome inhibitor LLnL prior to cell lysis, and ubiquitination of E2F1 was examined by sequential immunoprecipitation with anti-E2F1 antibody and immunoblotting with anti-HA antibody. E2F1 immunoprecipitates contained obvious high molecular weight smears detected by the anti-HA immunoblotting when cotransfected with either CUL1 (Fig. 3*B*, Lane 2) or CUL3 (Fig. 3*B*, Lane 4). These results confirm the recent report that overexpression of cullin 1 with E2F1 resulted in an increase of E2F1 ubiquitination *in vivo* (36). These results also provide *in vivo* evidence consistent with

<sup>5</sup> Manabu Furukawa, T. Ohta, and Y. Xiong, manuscript in preparation.

Fig. 4. *In vitro* E2F1 ubiquitination by ROC1-CUL1 does not require E2F1 phosphorylation or SKP1. **A**, CDK2-cyclin A enzyme was immunoprecipitated using anti-CDK2 antibody from lysate of insect cells coinfecting with baculoviruses expressing human CDK2 and cyclin A. Purified p21 protein was added to the lysate before anti-CDK2 precipitation (*Lane 1*). Purified E2F1 protein was incubated with anti-CDK2 immunoprecipitates (*Lanes 1 and 2*), with ROC1 immunoprecipitate from untransfected cells, or with HA-ROC1 precipitate from HA-ROC1- and cullin 1-transfected cells. After 30 min of incubation at 30°C in the presence of [ $\gamma$ - $^{32}$ P]ATP, reactions were terminated by adding SDS sample buffer containing 0.1 M DTT, then boiled for 5 min and resolved by SDS-PAGE before autoradiography. **B**, purified p21 protein was added to the total cell lysate before immunoprecipitation with anti-HA antibody (*Lane 1*). Purified E2F1 protein was incubated with HA immunocomplexes precipitated from HA-ROC1 and cullin 1 (*Lanes 1 and 2*) or vector pcDNA3 and cullin 1 (*Lane 3*) transfected cells. **C**, 293T cells were transiently cotransfected with HA-, ROC1-, and CUL1-expressing plasmids. Cell lysates were immunodepleted in three successive rounds with anti-HA-, anti-myc-, or anti-SKP1-coated protein A-agarose beads. Depletion was confirmed by immunoblotting. **D**, the lysates derived from 293T cells transfected with HA-ROC1 and CUL1 were immunodepleted with different antibodies and confirmed as described in **C**. The immunodepleted lysates were then precipitated with HA antibody and assayed for ubiquitination activity using purified E2F1 as substrate. **E**, 293T cells were cotransfected with myc3-ROC1 and either vector DNA control, wild type, or two HA-CUL1 mutants with impaired SKP1 binding. Myc3-ROC1/HA-CUL1 complex formation was purified with anti-HA antibody and incubated with purified E2F1 in the presence of E1, E2(Ubc5c), and ubiquitin. Ubiquitination of E2F1 was examined by anti-E2F1 immunoblotting.



the suggestion that multiple cullins are involved in E2F1 ubiquitination and that E2F1 can be ubiquitinated through a SKP1-independent manner, because other cullins including CUL3 do not interact with SKP1.

**Phosphorylation-independent *In Vitro* Ubiquitination of E2F1 by ROC1-CUL1.** Ubiquitination of all substrates of cullin 1/CDC53-dependent SCF ligase identified thus far is phosphorylation dependent (Refs. 6–8). Unphosphorylated E2F1 purified from bacteria, however, can be efficiently ubiquitinated by ROC1-cullin 1 (Fig. 1), leading us to test a phosphorylation-independent E2F1 ubiquitination. Although recombinant E2F1 purified from bacteria is not phosphorylated, there is a possibility that the ROC1-CUL1 immunocomplexes may contain a low level of E2F1 kinase activity that contributed to E2F1 ubiquitination. To eliminate this possibility, purified E2F1 protein was incubated in the presence of [ $\gamma$ - $^{32}$ P]ATP with ROC1 immunocomplexes derived from untransfected cells or with HA-ROC1 immunocomplexes derived from cells transfected with HA-ROC1 and cullin 1, and with a physiological E2F1 kinase, CDK2-cyclin A. Under the conditions where E2F1 can be readily phosphorylated by CDK2-cyclin A (Fig. 4A, *Lane 2*), neither ROC1 (Fig. 4A, *Lane 3*) nor HA-ROC1 immunocomplexes (Fig. 4A, *Lane 4*) catalyzed any detectable phosphorylation of E2F1. We further determined whether addition of CDK inhibitor p21 had any inhibitory effect on E2F1 ubiquitination by ROC1-CUL1. Purified p21 protein efficiently inhibited the E2F1 kinase activity of CDK2-cyclin A (Fig.

4A, *Lane 1*) but had no detectable effect on E2F1 ubiquitination by ROC1-CUL1 (Fig. 4B). Taken together, these results suggest that *in vitro* ubiquitination of E2F1 by ROC1-CUL1 does not depend on substrate phosphorylation. Phosphorylated E2F1 can also be ubiquitinated by ROC1-CUL1 (Fig. 1A), indicating that phosphorylation of E2F1 by CDK2-cyclin A does not inhibit the E2F1 ubiquitination either.

**SKP1-independent *In Vitro* Ubiquitination of E2F1 by ROC1-CUL1.** SKP1 functions as an adaptor molecule linking the cullin 1/CDC53 with an F box protein (5, 7, 8), thereby playing an essential role in mediating phosphorylated SCF-substrate interactions. SKP1, however, does not interact with other cullins (34). The finding that E2F1 can be ubiquitinated by ROC1-CUL1 *in vitro* in an apparently phosphorylation-independent manner and by multiple ROC-cullin ligases raises two possibilities: either E2F1 ubiquitination by other ROC-cullin ligases involves a yet to be identified SKP1-like molecule, or ROC1-CUL1 can catalyze E2F1 ubiquitination independent of SKP1. To test the latter hypothesis directly, we performed immunodepletion experiments. Extract was prepared from cells transiently transfected with HA-ROC1 and cullin 1 (untagged) and subjected to three consecutive rounds of immunodepletion with anti-HA antibody (depleting HA-ROC1), anti-myc antibody (negative control), or anti-SKP1 antibody. Depletion was confirmed by direct immunoblotting (Fig. 4C). Although depletion of HA-ROC1 completely removed E2F1 ubiquitin ligase activity (Fig. 4D, *Lane 1*), depletion of SKP1

(Fig. 4D, Lane 3), similar to the control anti-myc depletion (Fig. 4D, Lane 2), had no detectable effect on E2F1 ubiquitination by ROC1-CUL1 ligase.

To confirm further SKP1-independent *in vitro* E2F1 ubiquitination, we generated two mutant CUL1s that had significantly reduced (CUL1<sup>Y42A/M43A</sup>) and nearly completely disrupted (deletion of NH<sub>2</sub>-terminal 53 residues, CUL1<sup>ΔN53</sup>) SKP1 binding activity (confirmatory SKP1-CUL1 binding data not shown). Myc3-ROC1 was cotransfected into 293T cells with either wild-type or mutant HA-CUL1s. Individual ROC1-CUL1 complexes were purified by anti-HA antibody and assayed for E2F1 ubiquitin ligase activity *in vitro*. As shown in Fig. 4E, both CUL1<sup>Y42A/M43A</sup> (Lane 2) and CUL1<sup>ΔN53</sup> (Lane 3) mutants exhibited essentially the same level of ubiquitin ligase activity toward E2F1. Under the same assay condition, a ROC1 binding-deficient mutant CUL1 (CUL1<sup>Δ610-615</sup>) exhibited nearly undetectable E2F1 ubiquitin ligase activity (data not shown). Together, these results indicate that E2F1 can be ubiquitinated *in vitro* by ROC1-CUL1 ligase in a SKP1-independent manner.

## Discussion

In this report, we present the first evidence for *in vitro* E2F1 ubiquitination. Four lines of evidence corroborate that E2F1 ubiquitination by ROC-cullin ligases can be mediated by a mechanism distinct from that of the SCF: (a) E2F1 can be ubiquitinated by multiple ROC-cullin ligases, including cullins 2, 3, and 5, that do not interact with SKP1 (Fig. 3); (b) overexpression and inclusion of SKP1 and SKP2 had no detectable effect on E2F1 ubiquitination by ROC1-CUL1 (Fig. 1B). Under the same conditions, overexpression and inclusion of β-TrCP significantly enhanced IκBα ubiquitin ligase activity of ROC1-CUL1; (c) immunodepletion of SKP1 had no detectable effect on E2F1 ubiquitination by ROC1-CUL1 (Fig. 4D). Mutations in CUL1 that impair or disrupt SKP1 binding had no detectable effects on the level of E2F1 ubiquitin ligase activity of CUL1-ROC1 (Fig. 4E); and (d) unphosphorylated, recombinant E2F1 can be ubiquitinated by ROC-cullin ligases (Fig. 1B). Addition of p21 CDK inhibitor had no detectable effect on its ubiquitination (Fig. 4B).

The mechanism for targeting E2F1 ubiquitination by ROC-cullin ligases remains unclear. The detection of E2F1 *in vitro* ubiquitination may represent inefficient ligation or a low level of E2F1 targeting activity present in the ROC1 and cullin immunocomplex. This is similar to the case of IκBα ubiquitination, where IκBα was ubiquitinated at a low level by ROC immunocomplexes derived from untransfected cells but was significantly enhanced by the overexpression of β-TrCP (virtually all phosphorylated IκBα was ubiquitinated; Ref. 13). Although *in vitro* ubiquitination of E2F1 does not require E2F1 phosphorylation, our results do not suggest that phosphorylation of E2F1 is not involved in regulating its ubiquitination *in vivo*. For example, phosphorylation of E2F1 by a yet unidentified kinase on residues Ser-332 and Ser-337 has been reported to attenuate E2F1-Rb association (40) and could therefore indirectly regulate E2F1 ubiquitination *in vivo* by exposing uncomplexed E2F1 to ROC-cullin ligases. Establishment of *in vitro* ubiquitination of E2F1 makes it possible to biochemically purify the *in vivo* targeting activity and should facilitate the elucidation of the mechanism targeting the E2F1 ubiquitination *in vivo*.

## Acknowledgments

We thank Jiri Lukas for providing the GST-Rb expression plasmid, Dirk Bohmann for providing the ubiquitin expression vector, and Mike Tyers for sharing information on the SKP1-binding mutation of CUL1 before publication. We also thank Jen Michel, Manabu Furukawa, and Joe McCarville for

discussion throughout the work, critical reading of the manuscript, and figure preparation.

## References

- Jentsch, S. The ubiquitin-conjugating system. *Annu. Rev. Genet.*, 26: 179–207, 1992.
- Hochstrasser, M. Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.*, 30: 405–439, 1996.
- King, R. W., Deshaies, R. J., Peters, J.-M., and Kirschner, M. W. How proteolysis drives the cell cycle. *Science (Washington DC)*, 274: 1652–1659, 1996.
- Hershko, A. Role of ubiquitin-mediated proteolysis in cell cycle control. *Curr. Opin. Cell Biol.*, 9: 788–799, 1997.
- Bai, C., Sen, P., Hofmann, K., Ma, L., Goebel, 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.
- Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G., and Deshaies, R. J. Phosphorylation of Sic1p by G<sub>1</sub> Cdk required for its degradation and entry into S phase. *Science (Washington DC)*, 278: 455–460, 1997.
- Skowrya, 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.
- 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.
- 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.
- Mathias, N., Johnson, S. J., Winey, M., Adams, A. E. M., Goetsch, L., Pringle, J. R., Byers, B., and Gobel, M. G. Cdc53p acts in concert with cdc4p and cdc34p to control the G<sub>1</sub>-to-S phase transition and identifies a conserved family of proteins. *Mol. Cell Biol.*, 16: 6634–6643, 1996.
- 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.
- 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.
- Ohta, T., Michel, J. J., Schottelius, A. J., and Xiong, Y. ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Mol. Cell*, 3: 535–541, 1999.
- Tan, P., Fuches, S. Y., Angus, A., Wu, K., Gomez, C., Ronai, Z., and Pan, Z.-Q. Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of IκBα. *Mol. Cell*, 3: 527–533, 1999.
- Kamura, T., Koepp, D. M., Conrad, M. N., Skowrya, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science (Washington DC)*, 284: 657–661, 1999.
- Skowrya, D., Koepp, D. M., Kamura, T., Conrad, M. N., Conaway, R. C., Conaway, J. W., Elledge, S. J., and Harper, J. W. Reconstitution of G<sub>1</sub> cyclin ubiquitination with complexes containing SCF<sup>Grr1</sup> and Rbx1. *Science (Washington DC)*, 284: 662–665, 1999.
- Seol, J. H., Feldman, R. M. R., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K., and Deshaies, R. J. Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes Dev.*, 13: 1614–1626, 1999.
- Zachariae, W., and Nasmyth, K. Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev.*, 13: 2039–2058, 1999.
- Deshaies, R. J. SCF and cullin/RING H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.*, 15: 435–467, 1999.
- Tyers, M., and Jorgensen, P. Proteolysis and the cell cycle: with this RING I do thee destroy. *Curr. Opin. Genet. Dev.*, 10: 54–64, 2000.
- Honda, R., Tanaka, H., and Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.*, 420: 25–27, 1997.
- Joazeiro, C. A. P., Wing, S. S., Huang, H.-k., Levenson, J. D., Hunter, T., and Liu, Y.-C. The tyrosine kinase negative regulator c-Cbl as a RING-type E2-dependent ubiquitin-protein ligase. *Science (Washington DC)*, 286: 309–312, 1999.
- Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl. Acad. Sci. USA*, 96: 11364–11369, 1999.
- Levenson, J. D., Joazeiro, C. A. P., Page, A. M., Huang, H.-k., Hieter, P., and Hunter, T. The APC11 RING-H2 finger mediates E2-dependent ubiquitination. *Mol. Biol. Cell*, 11: 2315–2325, 2000.
- Gmachl, M., Gieffers, C., Podtelejnikov, A. V., Mann, M., and Peters, J.-M. The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex. *Proc. Natl. Acad. Sci. USA*, 97: 8973–8978, 2000.
- Dyson, N. The regulation of E2F by pRB-family proteins. *Genes Dev.*, 12: 2245–2262, 1998.
- Rebins, J. R. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. *Cell Growth Differ.*, 9: 585–593, 1998.
- Yamasaki, L., Jacks, T., Bronson, R., Gouillot, E., Harlow, E., and Dyson, N. J. Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell*, 85: 537–548, 1996.

29. Field, S. J., Tsai, F. Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell*, *85*: 549–561, 1996.
30. Hateboer, G., Kerkhoven, R. M., Shvarts, A., Bernards, R., and Beijersbergen, R. L. Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. *Genes Dev.*, *10*: 2960–2970, 1996.
31. Hofmann, F., Martelli, F., Livingston, D. M., and Wang, Z. The *retinoblastoma* gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. *Genes Dev.*, *10*: 2949–2959, 1996.
32. Campanero, M. R., and Flemington, E. K. Regulation of E2F through ubiquitin-proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. *Proc. Natl. Acad. Sci. USA*, *94*: 2221–2226, 1997.
33. Martelli, F., and Livingston, D. M. Regulation of endogenous E2F1 stability by the retinoblastoma family proteins. *Proc. Natl. Acad. Sci. USA*, *96*: 2858–2863, 1999.
34. Michel, J., and Xiong, Y. 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, 1998.
35. 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.
36. Marti, A., Wirbelauer, C., Scheffner, M., and Krek, W. Interaction between ubiquitin-protein ligase SCF<sup>SKP2</sup> and E2F1 underlies the regulation of E2F1 degradation. *Nat. Cell Biol.*, *1*: 14–19, 1999.
37. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. p27(KIP1) ubiquitination and degradation is regulated by the SCF (Skp2) complex through phosphorylated Thr187 in p27. *Curr. Biol.*, *17*: 661–664, 1999.
38. Carrano, A. C., Eyther, E., Hershko, A., and Pagano, M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nature Cell Biol.*, *1*: 193–199, 1999.
39. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. p45<sup>SKP2</sup> promotes p27<sup>KIP1</sup> degradation and induces S phase in quiescent cells. *Nat. Cell Biol.*, *1*: 207–214, 1999.
40. Fagan, R., Flint, K. J., and Jones, N. Phosphorylation of E2F-1 modulates its interaction with the *retinoblastoma* gene product and the adenoviral E4 p19 kDa proteins. *Cell*, *78*: 799–811, 1994.