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Phosphorylation- and SKP1-independent in Vitro Ubiquitination of E2F1 by Multiple ROC-Cullin Ligases

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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/Ubcc5 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 was 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 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.

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. Yinyon Ben-Neriah (The Hebrew University-Hadassah Medical School, Jerusalem, Israel). E2 Ubc5c was amplified from a
HeLa cDNA library by PCR and inserted into a T7 bacterial expression vector fused in-frame with a hexahistidine tag. A GST-Rb 379–928-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-6×His vector with isopropyl-1-thio-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 379–928 fusion protein was expressed in bacteria overnight at 25°C with isopropyl-1-thio-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% CO₂. Cell transfections were carried out using calcium-phosphate buffer. For each transfection, 15 or 45 μg 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 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 150 μg/ml benzamidine]. For immunodepletion, all steps were carried out at 4°C. One hundred μl 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 μl of each antibody-coated beads for three time periods (3 h, 3 h, and overnight) and once with 30 μl of uncoated protein A beads for 1 h to remove residual antibody. Ten μl of the lysate after depletion were subjected to direct Western, whereas 200 μl 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 coinfected with CDK2- and cyclin A-expressing baculoviruses using 3 μl 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 MgCl₂, 1 mM DTT, 0.4 mM Na₃VO₄, and 0.4 mM NaF], and added to 100 μl of kinase reaction mixture containing 10 μg of 6×His-E2F1 and 50 μCi of [γ⁻³²P]ATP. Reactions were incubated at 30°C for 30 min, and 5 μl of the supernatant were subjected to either SDS-PAGE or the ubiquitination assay. For the p21 kinase inhibition assay, 3 μg of purified 6×His-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 μg) antibody or from transfected cells with 3 μg of

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**In vitro ubiquitination of E2F1 by ROC1-CUL1.** A, purified E2F1 was phosphorylated and ³²P-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.
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 μl) containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 2 mM NaF, 10 mM okadaic acid, 2 mM ATP, 0.6 mM DTT, 60 ng of E1, 300 ng of E2, 0.5 μg of purified His-E2F1, and 12 μg of unlabelled 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 10 nM okadaic acid, 2 mM ATP, 0.6 mM DTT, 60 ng of E1, 300 ng of E2, 0.5 μg of purified His-E2F1, and 12 μg of unlabelled 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 mM 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 [32P]His-E2F1 phosphorylated by cyclin A-CDK2 enzyme. For Rb protection of E2F1 ubiquitination, 0.5 μg of purified His-E2F1 was first incubated with the indicated amounts of either GST or CUL1-Rb immunocomplexes in PBS buffer in a total volume of 15 μl 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 Coomassie Blue staining.

For in vivo ubiquitination assay, 293T cells on a 100-mm dish were transfected with appropriate plasmids expressing HA-UB (2.5 μg), GST-Rb (2.5 μg), CUL1 (10 μg), and CUL3 (10 μg). The total amount of plasmid DNA in each transfection was adjusted to a final 15 μg with pCDNA3 empty vector when needed. Thirty-six h after transfection, cells were treated with proteasome inhibitor LlL (50 μM) for 4 h. Cells were then collected, pelleted by centrifugation, lysed in 200 μl 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 μg). Immunoprecipitates were washed three times and resolved by 7.5% SDS-PAGE, followed by immunoblotting with anti-HA antibody (1 μg/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. IκBα, an inhibitor of the transcription factor NF-κB, and E2F1, a member of the E2F transcription factor family, were found to be efficiently ubiquitinated in vitro by the ROC-cullin immunocomplexes. SCFβTrCP-dependent ubiquitination of phosphorylated IκBα 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 [γ-32P]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 [32P] 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 [32P] 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, [32P]-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 ROC11349/Morra mutant that impairs the association of ROC1 with cullin 1 (34) or by the ROC1C75A/H77A 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, p45Skp2 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), p45Skp2 (Lane 7), or another F box protein, β-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
that overexpression of ROC1 and cullin 1 significantly enhanced E2F1 in vitro ubiquitination (Fig. 1B, Lane 6) and that E2F1 is in excess, these observations suggest that SKP2 (or β-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-Rb79–928 fusion protein (Fig. 2A) and confirmed its binding with E2F1 in vitro (Fig. 2B). When preincubated with E2F1, purified GST-Rb79–928, but not GST control, efficiently blocked in vitro ubiquitination of E2F1 by ROC1-CUL1 in a dose-dependent manner (Fig. 2C). 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. 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. Purified 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. 3A, Lane 2), CUL1 (Lane 3), myc-CUL2 (Lane 4), myc-CUL3 (Lane 5), myc-CUL5 (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. 3A, bottom panel), the lack of intrinsic activity (confirmatory data not shown), or a failure in complex assembly (Fig. 3A, 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 IkBα (data not shown). The myc-CUL5 immunocomplex derived from myc-CUL5 and HA-ROC1 cotransfected cells, although displaying E2F1 ligase activity (Fig. 3A, Lane 7), does not contain detectable HA-ROC1 (bottom panel). This observation suggests that CUL5 may prefer ROC2 as its ROC partner.

In vivo 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. 3B, Lane 2) or CUL3 (Fig. 3B, 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

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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 μM) 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.

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In vivo 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. 3B, Lane 2) or CUL3 (Fig. 3B, 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
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 coinfected 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 cyclin 1-transfected cells. After 30 min of incubation at 30°C in the presence of [γ-32P]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 cyclin 1 (Lanes 1 and 2) or vector pcDNA3 and cyclin 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 [γ-32P]ATP with ROC1 immunocomplexes derived from untransfected cells or with HA-ROC1 immunocomplexes derived from cells transfected with HA-ROC1 and cyclin 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 cyclin 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
terminal 53 residues, CUL1

Although should facilitate the elucidation of the mechanism targeting the E2F1

deficient mutant CUL1 (CUL1

ity toward E2F1. Under the same assay condition, a ROC1 binding-
mutants exhibited essentially the same level of ubiquitin ligase activ-
toward E2F1. Under the same assay condition, a ROC1 binding-
deficient mutant CUL1 (CUL1Y42A/M43A ) and nearly completely disrupted (deletion of NH 2 -

ubiquitination. Four lines of evidence corroborate that E2F1 ubiq-
thinstincted at a low level by ROC immunocomplexes derived from un-
fected into 293T cells with either wild-type or mutant HA-CUL1s.

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 ubiquiti-
nated at a low level by ROC immunocomplexes derived from un-
transformed 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 ubiqui-

inputing 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.

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 ubiqui-
tuin ligase activity of ROC1-CUL1; (c) immunodepletion of SKP1
had no detectable effect on E2F1 ubiquitination by ROC1-CUL1 (Fig. 1D). 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 is different from that of the SCF. In addition to the SCF E3
factor, Cdc4p, Skp1p, and Cdc53p/Cullin catalyzes ubiquitination of the phosphorylated

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