In vitro ubiquitination of cyclin D1 by ROC1–CUL1 and ROC1–CUL3

Ichiro Maeda[a,b], Tomohiko Ohta[a,*], Hirotaka Koizumi[b], Mamoru Fukuda[a]

[a] *Department of Surgery, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan*
[b] *Department of Pathology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan*

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Abstract Overexpression of cyclin D1 has been implicated in a variety of tumors, such as breast cancers, gastrointestinal cancers and lymphomas. Both gene amplification and protein degradation mediated by ubiquitin (Ub)-dependent proteolysis regulate the abundance of cyclin D1. Here we report that ROC1 interacted with all three D type cyclins in vivo but did not bind to other cyclins tested. The ROC1–CUL1 and ROC1–CUL3, but not ROC1–CUL2, –CUL3 and –CUL4, immunocomplexes promoted polyubiquitination of bacterially purified cyclin D1 in vitro. RING finger mutations of ROC1 eliminated the Ub ligase activity toward cyclin D1. In all cases the ubiquitination of cyclin D1 was accompanied by autoubiquitination of the cullins. The results suggest the involvement of ROC1–cullin ligases in cyclin D1 ubiquitination and a potential mechanism whereby the cullin subunit is ubiquitinated itself while ubiquitinating a substrate.

Key words: Cyclin D1; ROC1; Cullin; Ubiquitin ligase; SKP1–CUL1(CDC53)–F-box protein complex

1. Introduction

In mammalian cells mitogenic signals sequentially activate the transcription of D-type cyclins during G1 progression, resulting in the activation of cyclin-dependent kinase (CDK)4/6. Phosphorylation of Rb proteins by CDK4/6 releases the repression of E2F target genes and permits the G1/S transition [1]. While the levels of cyclins oscillate, the steady state levels of CDK proteins are relatively constant throughout the cell cycle as well as in cell cycle withdrawn terminally differentiated cells. Withdrawal of mitogens arrests cells in G1 primarily through cessation of D-type cyclin synthesis. Conversely, overexpression of D-type cyclins can overcome a mitogen-deprivation-imposed G1 arrest allowing abnormal entry into the cell cycle [2]. Supporting this notion, ectopic overexpression of cyclin D1 promotes cell cycle entry and causes cell transformation in vivo [3]. Two major mechanisms, the gene amplification of cyclin D1 and the increased protein stability of cyclin D1 have been linked with oncogenesis of different types of human cancers [4,5]. The protein stability of cyclin D1 is normally regulated by ubiquitin (Ub)-dependent proteolysis [6].

Ub-mediated proteolysis begins with activation of Ub in an ATP-dependent manner by a Ub-activating enzyme (E1) [7]. The charged Ub forms a high-energy thiolester bond with the E1 and is passed to a cysteine residue within an Ub-conjugating enzyme (E2). The E2-linked Ub is then transferred to a lysine residue in the substrate to form a terminal isopeptide bond, as targeted by a Ub ligase (E3). The proteins covalently conjugated with polyubiquitin chains are rapidly degraded by the 26S proteasome. Because the E3 provides substrate specificity, elucidating the mechanism and the regulation of E3 ligase activity have become critical issues central to our understanding of regulated proteolysis.

Previously, we and others identified a highly conserved small RING finger protein, ROC1 (also called Rbx1 and Hrt1), as an essential subunit of the SKP1–CUL1(CDC53)–F-box protein (SCF) Ub ligase [8–11]. The SCF and the anaphase-promoting complex (APC) are the two major Ub ligase complexes that regulate Ub-mediated proteolysis during G1/S and anaphase [12], and contain the small RING finger proteins ROC1 and APC11, respectively [8]. ROC1 commonly interacts with all cullins while APC11 specifically interacts with cullin-related APC2 [8]. Point mutations in the RING finger domain of ROC1 completely disrupted the Ub ligase activity, suggesting the domain's essential role in the activity [8]. ROC1–SCF catalyzes ubiquitination of phosphorylated IκBα from humans and G1 cyclin Cln2 and CDK inhibitor Sic1 from yeast [8–11]. Several studies support ROC1–SCF's ubiquitination of cyclin D1 in humans. The interaction between CUL1 and cyclin D1 in vivo was previously reported [13,14]. Treatment of cells with specific antisense oligonucleotides against either CUL1, SKP1 or SKP2 caused the accumulation of cyclin D1 protein [13]. However, the in vitro ubiquitination of cyclin D1 using these Ub ligases has not been shown. In this report we provide the evidence showing that ROC1–CUL1 and ROC1–CUL3 can catalyze cyclin D1 ubiquitination in vitro. We also demonstrate that the ubiquitination of cyclin D1 is accompanied by ubiquitination of the cullin subunit. The results suggest a potential mechanism whereby cullins are autoubiquitinated while targeting a substrate.

2. Materials and methods

2.1. Plasmids and antibodies

Constructs for human ROC1 and all cullins were previously described [8] except that three repeats of the myc epitope were linked

*Corresponding author. Fax: (81)-44-976 5964.
E-mail: to@marianna-u.ac.jp

Abbreviations: Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; SCF, SKP1–CUL1(CDC53)–F-box protein complex; APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; DTT, dithiothreitol

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for the myc-tagged cullins. Expression plasmids for cyclins were gifts from Dr. Yue Xiong (The University of North Carolina at Chapel Hill, NC, USA). ROC1 point mutations were introduced by site-directed mutagenesis (Stratagene) and verified by DNA sequencing. Mouse monoclonal anti-hemagglutinin (anti-HA; 12CA5, Boehringer-Mannheim), anti-myc (9E10, NeoMarkers) and anti-cyclin A (E72, NeoMarkers) antibodies were purchased commercially. Rabbit polyclonal antibodies recognizing a glutathione-*S*-transferase fusion full-length human cyclin B1 and polypeptides corresponding to the C-termini of human cyclins D1, D2, D3 and E1, were gifts from Dr. Yue Xiong. Rabbit anti-CUL1 antibody was previously described [8].

2.2. Cell culture, transfection, immunoprecipitation and immunoblotting

293T or Saos2 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Life Technologies) and 1% antibiotic–antimycotic agent (Life Technologies) in a 37°C incubator with 5% CO_2. Plasmid DNA was transfected using the standard calcium phosphate precipitation method (for 293T cells) or the LipofectAMINE (Gibco BRL) reagent according to the manufacturer's instructions (for Saos2 cells). For each transfection, the total plasmid DNA was adjusted to 5 or 15 μg per 60 or 100 mm dish, respectively, by adding the parental pcDNA3 vector if necessary. For coupled ^{35}S-labeling and immunoprecipitation, Saos2 cells cultured in 60 mm dishes were metabolically labeled with [^{35}S]methionine (0.15 mCi) 24 h after transfection, and then lysed and immunoprecipitated using 1 μg of anti-HA antibody or 1 μl of anti-cyclin sera as described elsewhere [15,16]. For the ROC1–cullin immunoprecipitation used in the in vitro Ub ligation assay, 293T cells were harvested 36 h after transfection and lysed by incubating at 4°C for 1 h with 0.6 ml per 100 mm dish of buffer A containing 15 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0.35% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 150 μg/ml benzamidine. Lysed cells were then clarified by centrifugation at 100 000×*g* at 4°C for 1 h. The supernatants (0.3 ml) were mixed with 3 μg of anti-myc or anti-HA antibody, and precipitated with protein A-agarose beads (Pierce; 7.5 μl). The proteins bound to the beads were used either for the Ub ligation assay or direct immunoblotting. The concentrations of the primary antibody used for the immunoblotting were anti-HA (1 μg/ml), anti-myc (1 μg/ml), anti-cyclin D1 serum (1:3000 dilution) and anti-CUL1 (1 μg/ml).

2.3. Enzymes and protein purification

Purified human cyclin D1 was a gift from Dr. Hideaki Higashi (Institute for Genetic Medicine, Hokkaido University). Purified rabbit E1 (Affiniti Research Products, Exeter, UK) and bovine Ub (Sigma) were purchased commercially. His-tagged UbcH5c and His-tagged mouse CDC34 were previously described [8,15].

2.4. Ub ligation assay

The ROC1–cullin immunocomplexes immobilized on protein A-agarose beads were washed three times with buffer A and twice with buffer containing 25 mM Tris–HCl pH 7.5, 50 mM NaCl, 0.01% NP-40, 10% glycerol and 1 mM EDTA, and added to an Ub ligation reaction mixture (final volume 30 μl) containing 50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 2 mM NaF, 10 nM okadaic acid, 2 mM ATP, 0.6 mM dithiothreitol (DTT), 40 ng E1, 300 ng E2, 50 ng of purified cyclin D1 and 12 μg of unlabeled bovine Ub. After incubation for 30 min at 37°C with shaking, the reactions were terminated by boiling in Laemmli sodium dodecyl sulfate (SDS)-loading buffer with 0.1 M DTT and half of the sample was resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblotting with either anti-cyclin D1, anti-CUL1 or anti-myc antibody.

3. Results

3.1. In vivo interaction of ROC1 with all D-type cyclins

In yeast the ubiquitination of G1 cyclin Cln2 is mediated by ROC1–SCF. To determine whether ROC1–SCF Ub ligases can be implicated in the ubiquitination of any human cyclins, we first tested the in vivo interaction between ROC1 and cyclins by examining immunocomplexes of ectopically expressed proteins. Saos-2 cells were transfected with plasmids

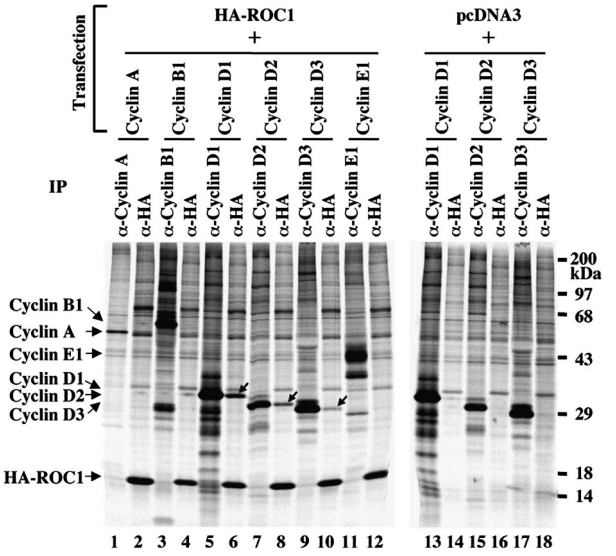

Fig. 1. Co-immunoprecipitation of D-type cyclins with ROC1. Saos2 cells cotransfected with the indicated cyclin plasmids and HA-ROC1 (left panel) or parental pcDNA3 plasmids (right panel) were metabolically labeled with [^{35}S]methionine. The ^{35}S-labeled proteins were immunoprecipitated with indicated antibodies and resolved by SDS–PAGE followed by autoradiography. Arrows indicate D-type cyclins coprecipitated with HA-ROC1 by the anti-HA antibody.

directing the expression of HA-tagged human ROC1 (HA-ROC1) together with either cyclin A, B1, D1, D2, D3 or E1. Transfected cells were metabolically labeled with [^{35}S]methionine, and cell lysates were immunoprecipitated with either α-HA, or antibody to each cyclin. All three D-type cyclins were co-precipitated with ROC1 by the α-HA antibody (Fig. 1, lanes 6, 8 and 10), but none of the other cyclins (lanes 2, 4 and 12). Replacement of pcDNA3-HA-ROC1 by the parental empty pcDNA3 vector eliminated the possibility of non-specific interaction between the anti-HA antibody and the D-type cyclins (lanes 14, 16 and 18). The results indicate that ROC1 can interact with D-type cyclins either directly, or indirectly through the complex associated with ROC1.

3.2. E1 and E2/UbcH5c-dependent ubiquitination of cyclin D1 by ROC1–CUL1 is accompanied by CUL1 autoubiquitination

The interaction between the ROC1 immunocomplex and the D-type cyclins prompted us to determine whether the cyclins are ubiquitinated by the ROC1 complex. We have previously shown that ROC1–CUL1 is capable of collaborating with two E2s, CDC34 and UbcH5c, to promote polyubiquitination [8]. Therefore, we first determined which E2 could collaborate with ROC1–CUL1 to promote cyclin D1 ubiquitination. An in vitro ubiquitination assay was performed using bacterially purified cyclin D1 protein, followed by detection with anti-cyclin D1 immunoblotting. Several slow migrating cyclin D1 species were detected when cyclin D1 was incubated with E1, E2/UbcH5c and HA-ROC1–CUL1 immunocomplexes (Fig. 2A, lane 2), which were not detected when UbcH5c was replaced with CDC34 (lane 1). Such slowly migrating products were promoted in an E1 (Fig. 2B, lane 1) and an E2/UbcH5c (lane 2)-dependent manner, indicating that they are ubiquitinated products. Incubation with HA immu-

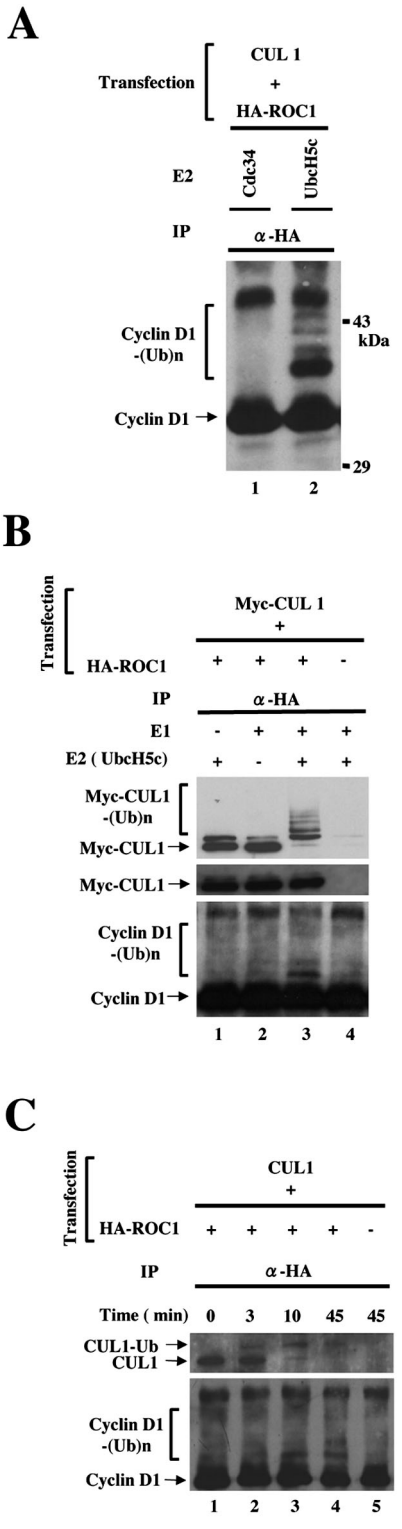

Fig. 2. In vitro ubiquitination of cyclin D1 by the ROC1–CUL1 immunocomplex. A: Purified cyclin D1 was incubated with Ub, E1, E2 (Cdc34 or UbcH5c as indicated) and anti-HA immunocomplexes derived from 293T cells transfected with HA-ROC1 and CUL1. The reactants were resolved by SDS–PAGE followed by anti-cyclin D1 immunoblotting. B: In vitro cyclin D1 ubiquitination was performed as in (A) except that myc-CUL1 was used instead of CUL1, with the combination of E1, E2/UbcH5c and HA-ROC1 as indicated. The ubiquitinated products of myc-CUL1 (top panel) and cyclin D1 (bottom panel) were detected with anti-myc and anti-cyclin D1 immunoblotting, respectively. The myc-CUL1 before the reaction was verified with anti-myc immunoblotting (middle panel). C: In vitro cyclin D1 ubiquitination was performed as in (A) with E2/UbcH5c for various lengths of time as indicated. The ubiquitinated products of CUL1 (upper panel) and cyclin D1 (lower panel) were detected with anti-CUL1 and anti-cyclin D1 immunoblotting, respectively. ←

nocomplexes from cells transfected with myc-CUL1 alone did not result in cyclin D1 ubiquitination (lane 4), excluding the possibility of a non-specific precipitation of the Ub ligase activity by the anti-HA antibody. The cyclin D1 polyubiquitination catalyzed by ROC1–CUL1 was time–course-dependent (Fig. 2C). Interestingly, when cyclin D1 was ubiquitinated, CUL1 (either myc-CUL1 or untagged CUL1) was simultaneously ubiquitinated (Fig. 2B, top panel, lane 3 and Fig. 2C, top panel, lanes 3 and 4). Ubiquitination of recombinant, unphosphorylated cyclin D1 suggests that the in vitro cyclin D1 ubiquitination by ROC1–CUL1 observed may not require substrate phosphorylation.

3.3. The RING finger of ROC1 is essential for the polyubiquitination of both cyclin D1 and CUL1

To seek direct evidence that the RING finger of ROC1 was essential for the observed Ub ligase activity directed toward either cyclin D1 or CUL1, we tested several point mutations of ROC1 for ligase activity. While ROC1$^{T9A/P10A}$, a mutation outside of the RING finger, did not affect the ubiquitination of cyclin D1 (Fig. 3, upper middle panel, lane 5), RING finger mutations of ROC1, ROC1$^{C53A/C56A}$ and ROC1$^{C75A/H77A}$, significantly reduced the ubiquitination (lanes 3 and 4) without disrupting the association of ROC1 with CUL1 (lower middle panel). The autoubiquitination of myc-CUL1 was also abolished by the RING finger mutations (top panel, lanes 2 and 5). These results indicate that the RING finger of ROC1 is essential for the polyubiquitination of both cyclin D1 and CUL1.

3.4. Cyclin D1 can be ubiquitinated by ROC1–CUL1 and ROC1–CUL3

ROC1 and ROC2 commonly interact with all the cullins and constitute a number of active Ub ligases. We next examined whether other ROC1–CUL complexes could catalyze the ubiquitination of cyclin D1. Purified recombinant cyclin D1 was incubated with myc immunocomplexes derived from cells transfected with HA-ROC and individual myc-tagged cullins. Two of five immunocomplexes tested, ROC1–CUL1 (Fig. 4, lower panel, lane 2) and ROC1–CUL3 (lane 4), were capable of catalyzing cyclin D1 ubiquitination in the presence of E1 and E2/UbcH5c. The cyclin D1 ubiquitination was not detected in myc-CUL2, myc-CUL4A or myc-CUL5 immunocomplexes (lanes 3, 5, and 6). The ubiquitination of cyclin D1 by ROC1–CUL1 and ROC1–CUL3 was accompanied by autoubiquitination of CUL1 and CUL3, respectively (upper panel, lanes 2 and 4).

4. Discussion

The in vivo interaction between ROC1 and cyclin D1, and the in vitro ubiquitination of cyclin D1 by ROC1–CUL1 and ROC1–CUL3 presented in this report indicate the potential of these complexes as intrinsic Ub ligases for cyclin D1. More

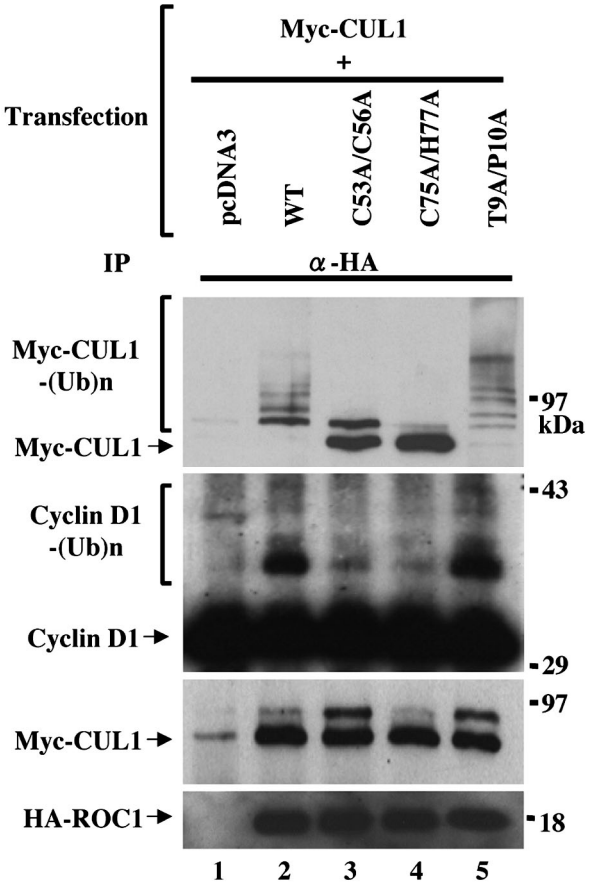

Fig. 3. The effect of Ub ligase activity on cyclin D1 and CUL1 in the ROC1–CUL1 immunocomplexes for various mutants of ROC1. 293T cells were co-transfected with myc-CUL1 and either vector DNA control, HA-tagged wild type, or HA-tagged ROC1 mutants as indicated. In vitro cyclin D1 ubiquitination was performed as in Fig. 1A with E2/UbcH5c and anti-HA immunocomplexes. The ubiquitinated products of myc-CUL1 (top panel) and cyclin D1 (upper middle panel) were detected with anti-myc and anti-cyclin D1 immunoblotting, respectively. ROC1–CUL1 complex formation was examined by coupled IP–Western (lower middle and bottom panel).

direct in vivo evidence is needed to firmly establish the function of ROC–cullin Ub ligases in targeting the ubiquitination and degradation of D-type cyclins. The in vitro assay of cyclin D1 ubiquitination reported here should facilitate future investigation into this issue. Two models present themselves to explain how the ROC1–CUL1 complex targets cyclin D1 for ubiquitination. One involves the ubiquitination of cyclin D1 through the ROC1–SCF complex, and the other is through a mechanism independent of SKP1 and F-box proteins. A common feature of the known F-box proteins, such as Cdc4 and β-TRCP, is the targeting of a phosphorylated substrate, such as Sic1 and IκBα, respectively [17,18]. Phosphorylation of cyclin D1 on threonine 286 by GSK-3β is required for the ubiquitination of cyclin D1 bound to CDK4 [6,19]. However, ubiquitination of free cyclin D1 can also occur independent of phosphorylation on threonine 286 [20]. The Ub ligase catalyzing the unphosphorylated substrate may be different from the one catalyzing phosphorylated, CDK4-bound cyclin D1. The bacterially purified cyclin D1 used in the present study was not phosphorylated, suggesting the ubiquitination of cyclin D1 was mediated by a non-SCF mechanism that does not require endogenous SKP1 and F-box proteins to be co-precipitated with the ROC1–CUL1 immunocomplex. Supporting this hypothesis, ROC1–CUL3, which does not interact with SKP1 [21], also showed Ub ligase activity with cyclin D1 as a substrate. Two different pathways exist for cyclin E1 ubiquitination as well. Cyclin E1 protein accumulated in mice deficient for SKP2 [22], but was also elevated in mice deficient for CUL3 [23]. CUL3 interacts with free, unphosphorylated cyclin E1 [23]. Such complementary mechanisms may generally exist for different substrates. Whether ROC1–CUL1 and ROC1–CUL3 utilize alternative unknown subunits to target the unphosphorylated substrate is not known. APC11 alone, without the presence of the cullin homolog APC2, could activate E2 and ubiquitinate known APC substrates, such as securin and cyclin B, in a destruction-box-independent manner, suggesting that substrate specificity may already exist at the level of RING finger proteins [24,25].

Our data clearly showed the ubiquitination of CUL1 and CUL3 in vitro. The ubiquitination of CUL1, as well as of its substrate cyclin D1, was dependent on the RING finger of ROC1. Other subunits of ROC1–SCF, the F-box proteins CDC4 and SKP2, have also been shown to be ubiquitinated, and the ubiquitination of SKP2 was mediated by a CUL1-based core Ub ligase complex [26,27]. The degradation of ROC1 has been shown to be proteasome-dependent [15]. These data together with the present data suggest that most of the subunits of the ROC1–SCF complex are autoubiquitinated. Whether the autoubiquitination has some specific role in substrate ubiquitination or is just a result of non-specific Ub ligase activity of the core catalytic subunit is not known. In the case of MDM2, a RING type Ub ligase for p53, mod-

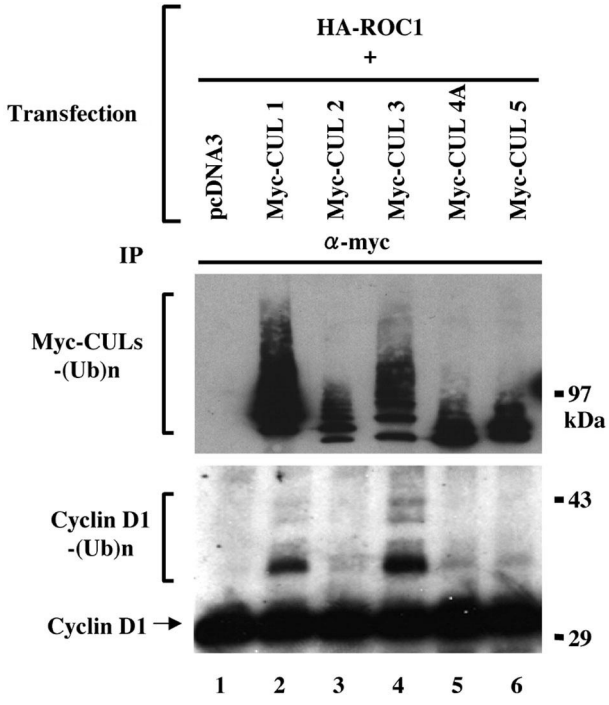

Fig. 4. In vitro ubiquitination of cyclin D1 by ROC1–CUL1 and ROC1–CUL3 Ub ligases 293T cells were co-transfected with HA-ROC1 and either vector DNA control or myc-tagged cullins as indicated. Individual ROC–cullin ligase complexes were precipitated with anti-myc antibody and incubated with cyclin D1 in the presence of E1, UbcH5c and Ub. The reaction mixture was resolved by SDS–PAGE followed by anti-myc (upper panel) or anti-cyclin D1 (lower panel) immunoblotting.

ification by SUMO-1 at K446 in the RING finger increases its Ub ligase activity toward p53 while protecting MDM2 from autoubiquitination and degradation [28]. A similar mechanism can be hypothesized for the ROC1–SCF Ub ligase complex. Further study is needed to elucidate the significance of the autoubiquitination of the ROC1–SCF subunits, including CUL1.

Cyclin D2 and cyclin D3 interact with ROC1 as well as cyclin D1, indicating that they may also be ubiquitinated by ROC1 containing Ub ligases. In breast cancer cell lines both cyclin D1 and cyclin D3 interact with CUL1, and the expression level of the two proteins is coordinately elevated, suggesting that cyclin D1 and cyclin D3 share a common mechanism of degradation [14]. Since the expression level of cyclin D is related to the potential for malignancy and the prognosis of a variety of cancers, revealing the mechanisms governing the Ub-dependent proteolysis of D-type cyclins is a critical issue in designing therapeutics for cyclin D-overexpressing cancers.

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