Autoinhibitory regulation of SCF-mediated ubiquitination by human cullin 1's C-terminal tail

Kosj Yamoah*, Taiji Oashi†, Antonio Sarikas*, Stefan Gazdoiu*, Roman Osman†, and Zhen-Qiang Pan*‡

Departments of *Oncological Sciences and †Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY 10029-6574

Communicated by Jerard Hurwitz, Memorial Sloan-Kettering Cancer Center, New York, NY, July 9, 2008 (received for review March 26, 2008)

SCF (Skp1·CUL1·F-box protein·ROC1) E3 ubiquitin ligase and Cdc34 E2-conjugating enzyme catalyze polyubiquitination in a precisely regulated fashion. Here, we describe biochemical evidence suggesting an autoinhibitory role played by the human CUL1 ECTD (extreme C-terminal domain; spanning the C-terminal 50 amino acids), a region that is predicted to contact the ROC1 RING finger protein by structural studies. We showed that ECTD did not contribute to CUL1's stable association with ROC1. Remarkably, deletion of ECTD, or missense mutations designed to disrupt the predicted ECTD·ROC1 interaction, markedly increased the ability of $SCF^{\beta TrCP2}$ to promote IκBα polyubiquitination and polyubiquitin chain assembly by Cdc34 *in vitro*. Thus, disruption of ECTD yields *in vitro* effects that parallel SCF activation by Nedd8 conjugation to CUL1. We propose that SCF may be subject to autoinhibitory regulation, in which Nedd8 conjugation acts as a molecular switch to drive the E3 into an active state by diminishing the inhibitory ECTD·ROC1 interaction.

autoinhibition | E3 ubiquitin ligase | neddylation

The SCF (Skp1·CUL1·F-box protein·ROC1) E3 ubiquitin (Ub) ligase defines a large class of evolutionally conserved four-subunit protein complexes that mediate timely, proteolytic destruction of a wide array of protein substrates, thereby regulating nearly all aspects of cellular and organismal processes (1). SCF is composed of an invariable core that contains Skp1, CUL1, and ROC1 (also named Rbx1 or Hrt1), and one interchangeable subunit belonging to the F-box protein family that consists of >70 members in humans. In SCF, CUL1 functions as a scaffold anchoring simultaneously an Skp1·F-box protein heterodimer and the ROC1 RING finger protein, hence positioning the substrate-bound F-box protein within the proximity of ROC1 that recruits an E2 Ub-conjugating enzyme. By mechanisms yet to be elucidated, the bound substrate, SCF and the E2 engage in concerted interactions that commit catalysis, yielding a covalent linkage connecting a polyubiquitin chain to a substrate lysine residue, which signals for proteolysis.

Initially, human SCF was noted to possess an intrinsic activity that stimulates the Cdc34 E2-conjugating enzyme for the synthesis of unanchored Ub chains (2, 3). Subsequent work has determined that this Ub ligase activity is mediated by ROC1 complexed with the C-terminal half of human CUL1 in a manner that strictly depends on the integrity of the RING finger domain (4, 5). Moreover, conjugation of Nedd8, an Ub-like molecule, to human CUL1 at K720, markedly enhanced the capability of Cdc34 to assemble substrate-free Ub chain (6), and to synthesize a di-Ub species (7, 8). Consistent with human reconstitution studies, Petroski and Deshaies (9) have demonstrated that in budding yeast SCF accelerates the discharge of Ub from Cdc34 and increases the rate of di-Ub formation. Taken together, it appears that the complex interactions between ROC1–CUL1–Nedd8 and an E2 (such as Cdc34) assemble an Ub polymerase activity. All SCF-related Cullin–RING ligases were found to contain Ub polymerization activity (3, 10, 11), suggesting a common mechanism used by these E3s for Ub catalysis.

In this study, we have uncovered an autoinhibitory activity by the CUL1 ECTD (extreme C-terminal domain; spanning amino acids 727–776) and propose that Nedd8 may exert its stimulatory role by reversing the ECTD-mediated inhibition.

Results

Reconstitution of Nedd8-Assisted Ubiquitination of IκBα by $SCF^{\beta TrCP2}$ and Cdc34. We sought to examine the influence of neddylation on the assembly of polyubiquitin chains onto IκBα by human Cdc34 E2-conjugating enzyme, because its counterpart in budding yeast plays an indispensable role in supporting SCF-mediated proteolysis (12). Our reconstituted *in vitro* ubiquitination system included GST-IκBα(1–54) phosphorylated by $IKK\beta^{S177E,S181E}$ as the substrate, Ub, E1, human Cdc34, and the four-subunit $SCF^{\beta TrCP2}$ E3 complex. To evaluate the role of neddylation, we used two experimental approaches, with the first procedure involving the initial assembly of the $SCF^{\beta TrCP2}$–IκBα complex, followed by treatment with the neddylation agents and then, the addition of ubiquitination enzymes (Fig. 1*A*, approach 1). As shown, $SCF^{\beta TrCP2}$, containing CUL1 with ≈10% in neddylated form as revealed by immunoblot analysis (Fig. 1*B Lower*, lanes 2–6), supported the ubiquitination of ^{32}P-GST-IκBα(1–54), producing laddered forms of substrate-Ub conjugates (Fig. 1*B Upper*, lanes 2–5), a majority of which ranged in size from 100 to 250 KDa (Fig. 1*B Upper*, lane 5). However, incubation of $SCF^{\beta TrCP2}$-IκBα with neddylation agents converted the majority of CUL1 from unmodified to neddylated forms (Fig. 1*B Lower*, lanes 7–11). The resulting hyperneddylated $SCF^{\beta TrCP2}$ produced a heterogeneous population of ^{32}P-GST-IκBα(1–54)-Ub conjugates of molecular mass from 150 to far beyond 250 kDa, considerably longer than those formed with the underneddylated E3 (Fig. 1*B Upper*, compare lanes 4 and 5 with lanes 9 and 10). Notably, the extensive ubiquitination required both neddylation and a high concentration of Cdc34 (Fig. 1*B Upper*, lanes 9 and 10). Unexpectedly, this neddylation procedure also formed a ^{32}P-GST-IκBα(1–54) conjugate independently of Cdc34 (Fig. 1*B Upper*, lanes 7, 8, and 11), which matched the size of mononeddylated substrate. Intriguingly, this mononeddylated species disappeared in the presence of high levels of Cdc34 (Fig. 1*B upper*, lanes 9 and 10), suggesting that Cdc34 was able to use this modified substrate for Ub chain assembly *in vitro*. On a cautious note, although our observations suggest enzymatic capability by human Cdc34 in assembling Ub chains on Nedd8, its biological implication is not understood at the present time.

To evaluate IκBα polyubiquitination exclusively, we used approach 2 to eliminate substrate mononeddylation, by treating $SCF^{\beta TrCP2}$ with neddylation agents first, and then washing the complex before the addition of substrate (Fig. 1*A*, approach 2). Under this condition, mononeddylation of ^{32}P-GST-IκBα(1–54)

Author contributions: K.Y. and Z.-Q.P. designed research; K.Y. and A.S. performed research; K.Y., T.O., A.S., S.G., and R.O. contributed new reagents/analytic tools; K.Y., T.O., R.O., and Z.-Q.P. analyzed data; and K.Y. and Z.-Q.P. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

‡To whom correspondence should be addressed. E-mail: zhen-qiang.pan@mssm.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0806155105/DCSupplemental.

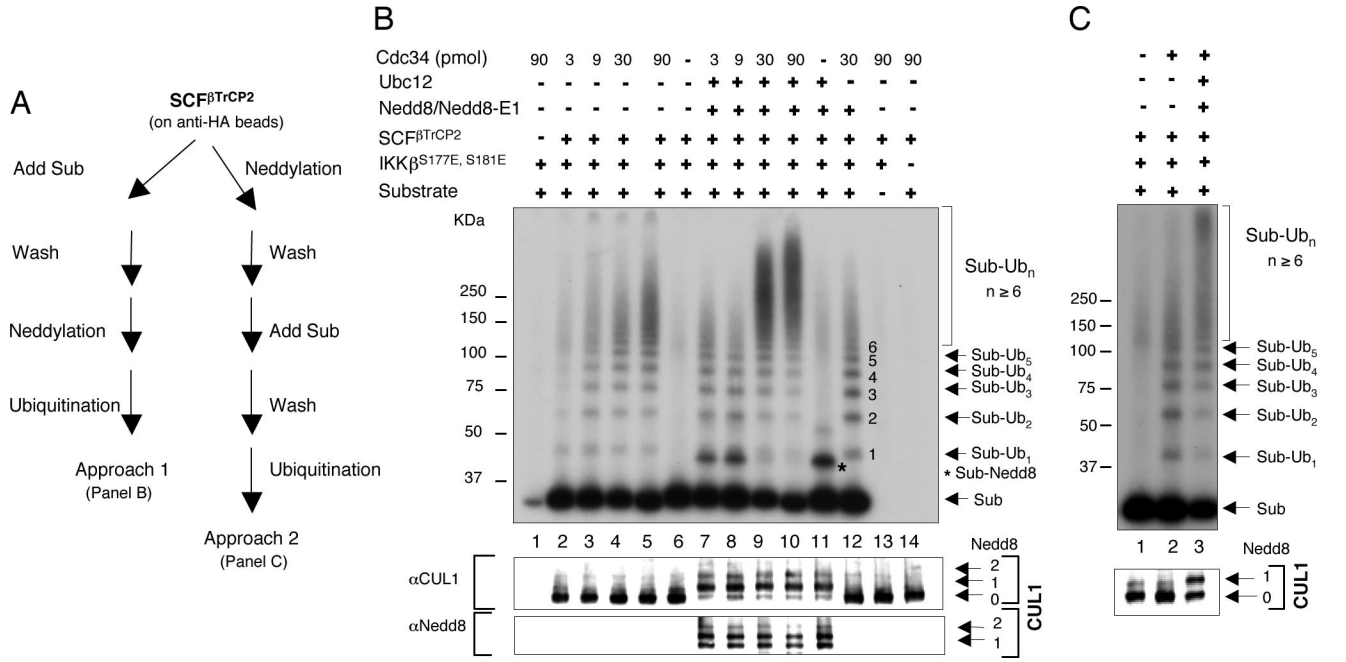

Fig. 1. Neddylation stimulates SCF$^{\beta TrCP2}$/Cdc34-mediated ubiquitination of IκBα. (*A*) A scheme of experimental procedures used for the *in vitro* ubiquitination of IκBα by SCF$^{\beta TrCP2}$. (*B* and *C*) Reconstitution of Nedd8-assisted ubiquitination of GST-IκBα(1–54) by SCF$^{\beta TrCP2}$ and Cdc34 using approach 1 or 2, respectively. Ubiquitination was carried out as described in *SI Text*. Both autoradiogram and immunoblots with indicated antibodies are shown. The numbers in the middle of the autoradiogram denotes the suggested number of Ub moieties conjugated to the substrate. * marks the conjugate formed by the substrate and Nedd8. Note the multiple CUL1-Nedd8 species shown in *B*. As revealed by recent mass spectrometry studies, Nedd8 K11, K22, K48, and K60 can form chains *in vivo*, whereas K22 and K48 can be neddylated *in vitro* (25). The biological significance of the Nedd8 chains remains to be elucidated.

did not occur (Fig. 1*C Upper*, lane 3). Nonetheless, the hyperneddylated SCF$^{\beta TrCP2}$ was found to be more effective than the underneddylated E3 in producing Ub conjugates with molecular mass far >250 kDa (Fig. 1*C Upper*, compare lanes 2 and 3). Collectively, these findings demonstrate a prominent role for neddylation in promoting extensive ubiquitination of IκBα by human Cdc34.

***In Vitro* Activation of SCF by Deletion of Human CUL1 ECTD.** We investigated the role of human ECTD (Fig. 2*A*) in SCF-mediated ubiquitination. ECTD defines a highly conserved region spanning residues 727–776, comprising α-helices H30 and H31 and β-strands S10 and S11 within the CUL1 winged-helix B domain (13). As revealed by crystallographic analysis, the human CUL1 ECTD forms extensive contacts with the ROC1 RING finger protein (13), which involve ROC1 H3 helix and S2 strand, as well as CUL1 H31 helix and S10 strand (Fig. 2*B*). Specifically, CUL1 Y761 and E758 form hydrogen bonds with ROC1 K89, S85, and T64. Significantly, substitution of yeast ROC1 at the corresponding K89 and R91 positions with alanine failed to rescue ΔRbx1/ROC1 yeast strain (13), underscoring a role for these residues in mediating interactions of critical functional significance.

Coexpression of CUL1$^{324–728}$, a human CUL1 ECTD deletion mutant, with ROC1 in bacteria formed a complex with efficiency comparable to that of the WT [supporting information (SI) Fig. S1*A*], demonstrating that ECTD does not contribute to CUL1's stable association with ROC1. However, ECTD deletion rendered CUL1 deficient in neddylation *in vitro* (Fig. S1*B*) and *in vivo* (Fig. S1*C*), which is in complete agreement with the previous observations with Cdc53 (14) and human CUL1 (15).

To determine the effects of ECTD deletion in the ubiquitination of IκBα, we isolated SCF$^{\beta TrCP2}$ or the mutant E3 containing CUL1$^{1–728}$, which were assembled in FCHT293 and FC728HT293 cells, respectively, by using a double-affinity purification approach as described in *SI Text*. Immunoblot analysis revealed comparable amounts of HA-βTrCP2 and Flag–CUL1 or Flag–CUL1$^{1–728}$, associated with SCF$^{\beta TrCP2}$ and SCF$^{\beta TrCP2}$ (CUL1$^{1–728}$), respectively (Fig. 3*A Lower*). Whereas ≈10% of CUL1 was found neddylated (Fig. 3*A Lower*, lanes 3 and 4),

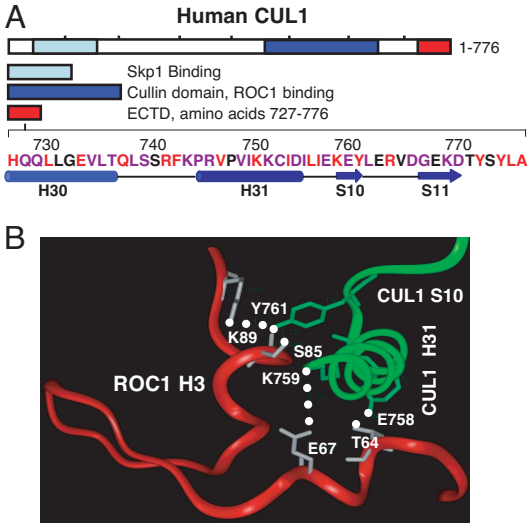

Fig. 2. The human CUL1 ECTD. (*A*) A schematic representation of domains of the human CUL1. ECTD comprises C-terminal amino acids 727–776 (downstream of the neddylation site (K720), with secondary structures as previously determined (13), and with identical residues among CUL1 homologs and paralogs colored in red and conserved amino acids in purple. (*B*) The interface structure between the CUL1 ECTD H31 helix and S10 β-strand (green), as well as the ROC1/Rbx1 H3 helix (red). This structure was generated based on the crystal structure of the CUL1–Rbx1–Skp1–F-box^{Skp2} complex (13), as described in *SI Text*. Residues involved in direct interface contact are indicated, with white dotted lines denoting hydrogen bonds and salt bridges.

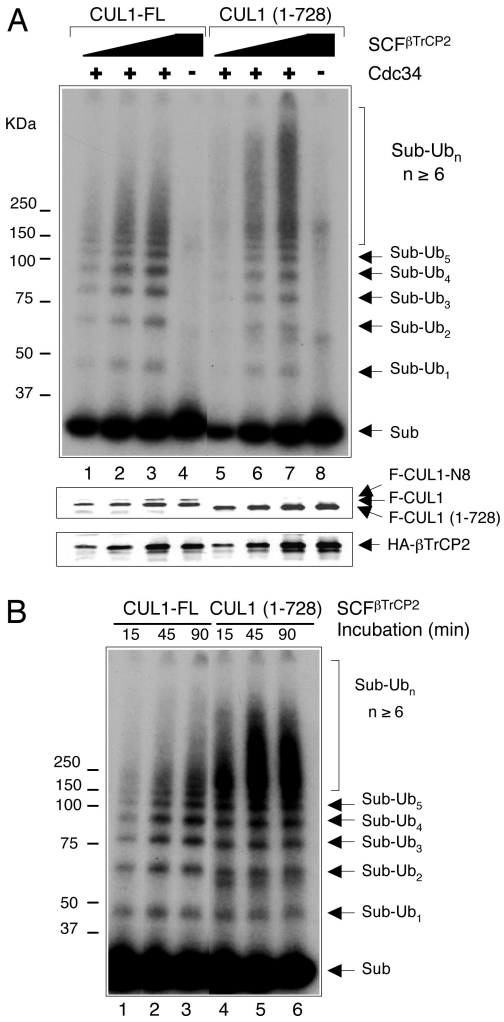

Fig. 3. Deletion of the CUL1 ECTD activates the ubiquitination of IκBα by $SCF^{\beta TrCP2}$/Cdc34. (*A*) Titration. Increasing amounts of $SCF^{\beta TrCP2}$ and $SCF^{\beta TrCP2}$ ($CUL1^{1-728}$), prepared as described in *SI Text*, were compared for their ability to support the ubiquitination of IκBα with Cdc34 (90 pmol). The reaction was analyzed by autoradiogram (*Upper*), to visualize ^{32}P-GST–IκBα (1–54)–Ub conjugates, and by immunoblot (*Lower*), to reveal the levels of HA–βTrCP2 and Flag-tagged CUL1 or mutant in each E3 complex used. (*B*) Reaction kinetics. The amounts of $SCF^{\beta TrCP2}$ and $SCF^{\beta TrCP2}$ ($CUL1^{1-728}$) used were identical to those used in reactions shown in *A*, lanes 2 and 6, respectively.

$CUL1^{1-728}$ was unmodified exclusively (Fig. 3*A* *Lower*, lanes 5–8), as expected.

Surprisingly, $SCF^{\beta TrCP2}$ ($CUL1^{1-728}$) was significantly more active than the underneddylated WT E3 complex in mediating the ubiquitination of ^{32}P-GST–IκBα(1–54) (Fig. 3*A* *Upper*, lanes 6 and 7) in a manner that depends on Cdc34 (Fig. 3*A* *Upper*, lane 8). Quantitation analysis using a phosphorimager revealed that $SCF^{\beta TrCP2}$ ($CUL1^{1-728}$) yielded GST–IκBα(1–54)–Ub conjugates of >250 kDa in amounts about four times higher than those observed with the WT E3. Furthermore, kinetic analysis showed that $SCF^{\beta TrCP2}$ ($CUL1^{1-728}$) accumulated more high-molecular-mass ^{32}P-GST–IκBα(1–54)-Ub species at 15 min than $SCF^{\beta TrCP2}$ did after 90 min of incubation (Fig. 3*B*, compare lanes 3 and 4). Thus, $SCF^{\beta TrCP2}$ ($CUL1^{1-728}$) resembled the WT E3 complex containing hyperneddylated CUL1, in producing extensively ubiquitinated products (compare Fig. 3*A*, lanes 6 and 7, and Fig. 3*B*, lanes 4–6, with Fig. 1*B*, lanes 9 and 10, and Fig. 1*C*, lane 3). Taken together, these results strongly suggest that ECTD deletion enhances the ability of $SCF^{\beta TrCP2}$ to promote the ubiquitination of IκBα.

Next, we examined the influence of ECTD truncation in Ub chain assembly by the ROC1–CUL1 subcomplex. Kinetic analysis revealed that ROC1–$CUL1^{324-728}$, deleted of the CUL1 ECTD, exhibited much higher Ub polymerization activity than the unmodified WT complex (Fig. 4*A*, lanes 1–14), stimulating the production of free Ub chains (di-Ub or tri-Ub) as well as polyubiquitinated Cdc34 by 4- to 5-fold (Fig. 4*A*, graphs). In agreement with previous studies (6, 7), neddylation of $CUL1^{324-776}$ markedly enhanced polyubiquitin chain assembly (Fig. 4*A*, compare lanes 17 and 18). Importantly, the activity by the unmodified ROC1–$CUL1^{324-728}$ was similar to that observed with ROC1–$CUL1^{324-776}$–Nedd8, as judged by similar production of di-Ub, tri-Ub, and polyubiquitinated Cdc34 by these two complexes (Fig. 4*A*, compare lanes 18 and 19). As shown, addition of neddylation components did not stimulate ubiquitination by the ECTD-truncated complex (Fig. 4*A*, compare lanes 19 and 20). In addition, ROC1–$CUL1^{324-728}$ was found to be more active than ROC1–$CUL1^{324-776}$ to assemble Ub chains in the range of Cdc34 concentrations examined (Fig. S2). Altogether, these data demonstrated that removal of the CUL1 ECTD stimulated human Cdc34 for the synthesis of polyubiquitin chains.

To characterize precisely the effect of ECTD on Ub ligation by Cdc34, we measured the synthesis of di-Ub species formed via the ligation of ^{32}P-labeled Ub^{K48R} to bovine Ub, as described (7). Consistent with previous observations (7), in the absence of Nedd8, ROC1–$CUL1^{324-776}$ supported the formation of di-Ub poorly (Fig. 4*B*, compare lane 2 with lanes 3–5). In contrast, ROC1–$CUL1^{324-728}$ markedly increased di-Ub synthesis in a concentration dependent manner (Fig. 4*B*, lanes 6–8, and graph). Taken together, these results concluded that the deletion of ECTD promoted extensive ubiquitination of IκBα by enhancing the ability of human Cdc34 to catalyze the assembly of polyubiquitin chains. Hence, the ECTD truncation appeared to phenocopy neddylation in stimulating ubiquitination.

Disruption of the Human CUL1 ECTD·ROC1 Interface Activates SCF *in Vitro*. Given that the human CUL1 ECTD is dispensable for stable association with ROC1 (Fig. S1*A*) but its removal activated ubiquitination (Figs. 3 and 4), we postulated that the ECTD·ROC1 interface might play an autoinhibitory role. To test this hypothesis, we sought to disrupt the predicted ECTD·ROC1 interface by creating alanine substitution at CUL1 Y761 or ROC1 K89. To avoid neddylation-mediated effects, we generated Flag–$CUL1^{K720R,Y761A}$ bearing both K720R and Y761A mutations. Using procedures described in *SI Text*, varying amounts of $SCF^{\beta TrCP2}$, $SCF^{\beta TrCP2}$ ($CUL1^{K720R}$), or $SCF^{\beta TrCP2}$ ($CUL1^{K720R,Y761A}$) were affinity-purified, with each set containing comparable amounts of HA–βTrCP2 and Flag–CUL1 or mutants, as confirmed by immunoblot analysis (Fig. 5*A*). As expected, whereas the WT Flag–CUL1 contained ≈10% neddylated form (Fig. 5*A* *Upper*, lanes 1 and 2), no detectable neddylation was observed with $CUL1^{K720R}$ or $CUL1^{K720R,Y761A}$ (Fig. 5*A* *Upper*, lanes 3–6). $SCF^{\beta TrCP2}$ supported the ubiquitination of ^{32}P-GST–IκBα(1–54) more effectively than $SCF^{\beta TrCP2}$ ($CUL1^{K720R}$) (Fig. 5*B*, compare lanes 1 and 2 with lanes 8–10), in keeping with a stimulating role for neddylation as demonstrated (16–18). Importantly, $SCF^{\beta TrCP2}$ ($CUL1^{K720R,Y761A}$) supported the formation of high-molecular-mass ^{32}P-GST–IκBα(1–54)–Ub conjugates more effectively than $SCF^{\beta TrCP2}$ ($CUL1^{K720R}$) (Fig. 5*B*, compare lanes 4–6 with lanes 8–10), in levels similar to the WT E3, especially when low levels of SCF were used (Fig. 5*B*, graph). Thus, introduction of a single Y761A substitution into $CUL1^{K720R}$ was able to reverse the inhibition caused by deficient neddylation.

Next, we examined the effects of the CUL1 Y761A or ROC1

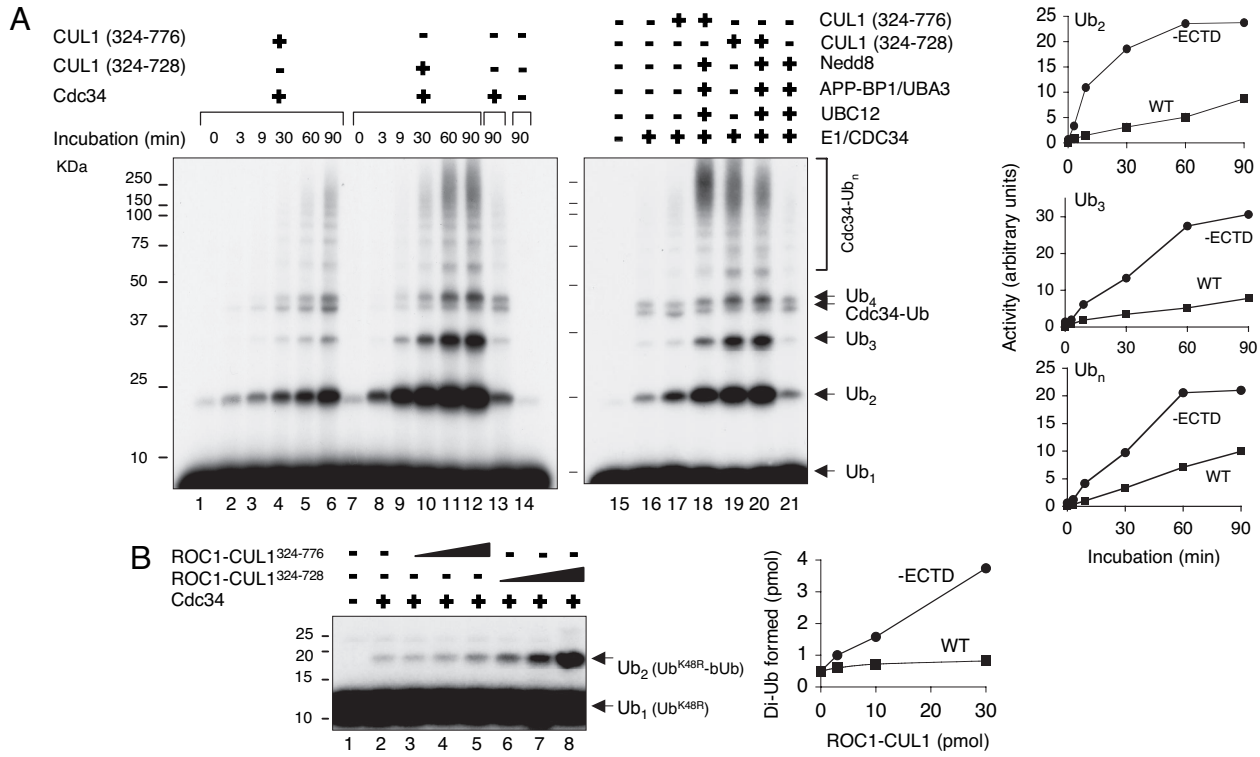

Fig. 4. Truncation of the CUL1 ECTD promotes polyubiquitin chain assembly catalyzed by human Cdc34. (*A*) Polyubiquitin chain assembly. The Ub polymerization assay was carried out as described in *SI Text*. A time course experiment is shown (lanes 1–14), using ROC1-CUL1$^{324-776}$ and ROC1-CUL1$^{324-728}$ (30 pmol each). Lane 13 omitted the ROC1–CUL1 complex. Lane 14 contained Ub and E1 only. The reaction products were analyzed by autoradiography and quantitated by PhosphoImager, with the synthesis of Ub_2, Ub_3, or Cdc34-Ub_n, presented graphically. The effects of neddylation were examined in reactions shown in lanes 15–21. In this case, ROC1-CUL1$^{324-776}$ or ROC1-CUL1$^{324-728}$ was treated with neddylation agents as described in *SI Text*, followed by the addition of E1, Cdc34, and ^{32}P-Ub to initiate polyubiquitin chain assembly reaction. The incubation time was 90 min. Similar levels of CUL1$^{324-776}$ and CUL1$^{324-728}$ were used (Fig. S6*A*). (*B*) di-Ub synthesis. ROC1-CUL1$^{324-776}$ and ROC1-CUL1$^{324-728}$ (30 pmol each) were compared for their ability to support Cdc34-catalyzed di-Ub synthesis, measured as described in *SI Text*. The reaction was incubated for 60 min at 37°C with Cdc34 (30 pmol). Formation of di-Ub was quantitated and graphically presented. Similar levels of CUL1$^{324-776}$ and CUL1$^{324-728}$ were used (Fig. S6*B*).

K89A mutation in the assembly of Ub chain by human Cdc34. Supporting our prediction, the purified ROC1–CUL1 (Y761A)$^{324-776}$ complex was significantly more active than the WT complex in polyubiquitin chain assembly by Cdc34 (Fig. 5*C Upper*, compare lanes 3 and 4 with lanes 5 and 6). As shown, CUL1 (Y761A)$^{324-776}$ was efficiently neddylated (Fig. 5*C Bottom*, compare lanes 7 and 8). However, neddylation of CUL1 (Y761A)$^{324-776}$ did not further increase the Ub polymerization activity (Fig. 5*C Upper*, compare lanes 6 and 8). Moreover, the activity by ROC1–CUL1 (Y761A)$^{324-776}$ was similar to that observed with ROC1–CUL1$^{324-776}$–Nedd8 (Fig. 5*C*, compare lanes 6 and 8). Similar effects were also observed with ROC1–CUL1 (Y761A)$^{324-776}$ and ROC1–CUL1$^{324-776}$ at lower concentrations in the presence or absence of neddylation (Fig. S3). In addition, the reaction kinetics of polyubiquitin chain assembly by ROC1–CUL1 (Y761A)$^{324-776}$ paralleled that by the neddylated WT complex (data not shown). Similar stimulatory effects were observed with the ROC1 K89A substitution (Fig. S4). Furthermore, both ROC1–CUL1 (Y761A)$^{324-776}$ and ROC1 (K89A)–CUL1$^{324-776}$ supported the synthesis of di-Ub more effectively than the WT complex (Fig. S5).

Altogether, these results demonstrated that single missense mutations, created to disrupt the predicted ECTD·ROC1 interface, enhanced the ability of SCF$^{\beta TrCP2}$ and ROC1–CUL1 to support substrate ubiquitination and polyubiquitin chain assembly, respectively. These effects resembled those observed with the ECTD deletion mutant (Figs. 3 and 4), thus supporting the hypothesis that the CUL1 ECTD·ROC1 interaction inhibits ubiquitination catalyzed by human Cdc34. Of note, the extent of the activation by single missense mutations was less robust than that seen with the ECTD deletion mutant, most likely caused by incomplete disruption of the CUL1 ECTD·ROC1 interface.

Discussion

Previously, the role of CUL1 has been defined as a scaffold that tethers both Skp1 and ROC1 and provides a site (K720 in human CUL1) for Nedd8 conjugation, which leads to activated ubiquitination. In this work, we have provided compelling biochemical evidence suggesting an autoinhibitory role played by the human CUL1 ECTD (Fig. 2*A*) in polyubiquitination catalyzed by Cdc34. We showed that: (*i*) ECTD did not contribute to the stable association between CUL1 and ROC1 (Fig. S1*A*); (*ii*) ECTD deletion markedly increased the ability of SCF$^{\beta TrCP2}$ to promote the *in vitro* ubiquitination of IκBα (Fig. 3) and polyubiquitin chain assembly (Fig. 4); and (*iii*) single mutations that were designed to disrupt the predicted ECTD·ROC1 interaction, such as CUL1 Y761A and ROC1 K89A, activated SCF$^{\beta TrCP2}$ for IκBα polyubiquitination and the assembly of polyubiquitin chains (Fig. 5 *A–C* and Figs. S4 and S5).

These findings led us to hypothesize that the human CUL1 ECTD acts as an allosteric module to regulate Cdc34-catalyzed polyubiquitin chain assembly. In this model, the interface between ECTD and ROC1 (Fig. 2*B*) represents a "restrained" state that suppresses Cdc34 activity. Disruption of the ECTD module through deletion or missense mutation converts this "latent" conformation into an active form. Previous studies have

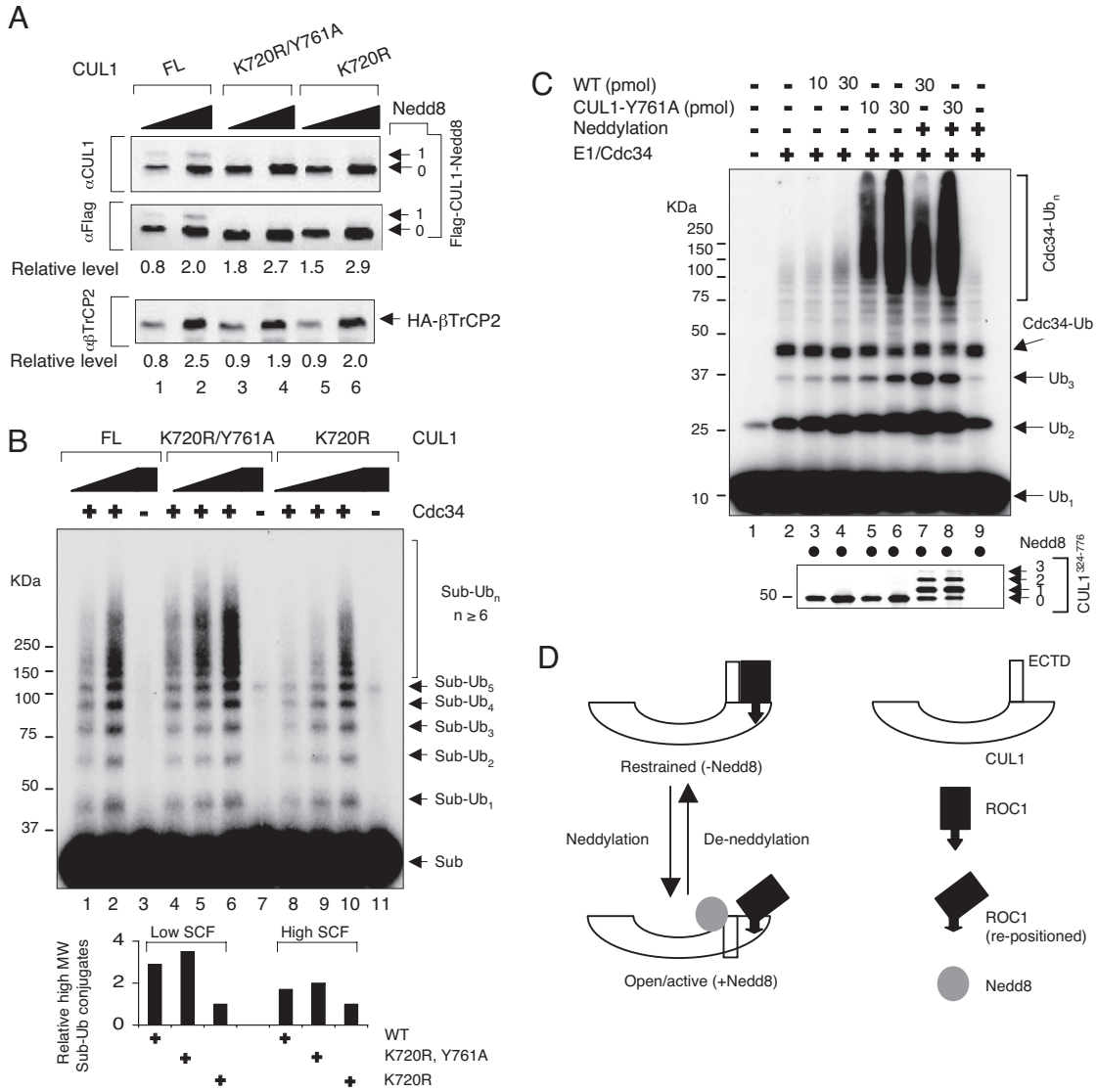

Fig. 5. Activation of SCF$^{\beta TrCP2}$-mediated ubiquitination of IκBα by CUL1 Y761A substitution. (*A*) Immunoblot analysis. SCF$^{\beta TrCP2}$, SCF$^{\beta TrCP2}$ (CUL1^{K720R}), or SCF$^{\beta TrCP2}$ (CUL1$^{K720R/Y761A}$) was prepared as described in *SI Text*. The levels of HA–βTrCP2 and Flag–CUL1 or mutant (Flag–CUL1^{K720R} or Flag–CUL1$^{K720R/Y761A}$) in isolated E3s were determined by immunoblot analysis and quantitated by the Odyssey infrared imaging system (Licor). As revealed by the numbers displayed, each immuno-purified complexes contained comparable levels of the SCF subunits. In addition, quantitative immunoblot analysis indicated that anti-HA immunoprecipitation copurified predominantly Flag–CUL1 or mutant (with the ratio of recombinant against the endogenous >4:1; data not shown). (*B*) Autoradiographic analysis of *in vitro* ubiquitination of IκBα. The amount of purified SCF$^{\beta TrCP2}$ used in lanes 1 and 2, SCF$^{\beta TrCP2}$ (CUL1$^{K720R/Y761A}$) in lanes 5 and 6, and SCF$^{\beta TrCP2}$ (CUL1^{K720R}) in lanes 9 and 10, were identical to those in Fig. 5*A*, lanes 1–6. The amount of SCF$^{\beta TrCP2}$ (CUL1$^{K720R/Y761A}$) used in lane 4, and SCF$^{\beta TrCP2}$ (CUL1^{K720R}) in lane 8, was 3-fold less than that in lane 5 and lane 9, respectively. The production of GST–IκBα(1–54)–Ub conjugates of >150 kDa by the three SCF$^{\beta TrCP2}$ complexes was quantitated by PhosphorImager, and the ratio between the WT or the K72OR/Y761A mutant against K72OR was presented graphically. (*C*) Effects of the CUL1 Y761A mutation in polyubiquitin chain assembly. ROC1–CUL1$^{324-776}$ and ROC1–CUL1 (Y761A)$^{324-776}$ (30 pmol each) were compared for their ability to support Cdc34-catalyzed polyubiquitin chain assembly in the presence or absence of neddylation, as described in Fig. 4*A*. (*D*) A model for role of the CUL1 ECTD in autoinhibitory regulation of SCF. In the absence of Nedd8, the CUL1 ECTD makes contacts with ROC1, forming a restrained/inactive conformation. It is proposed that neddylation may disrupt the ECTD–ROC1 interaction to create an active state. The arrow in black denotes the N terminus of ROC1 that establishes primary interface interactions with CUL1 (13).

suggested allosteric regulation of an E3 activity, with examples including activation of the Ubr1 E3 by dipeptides (19), and C2 domain-mediated autoinhibition of Smurf2 HECT E3 ligase (20). Allosteric regulation of an enzymatic activity is well documented with studies of c-Src, whereby the kinase domain is kept in an inactive conformation by multiple interactions involving Src homology 3 (SH3), phospho-tyrosine-bound SH2, and the linker that connects the kinase and SH2 domains (21). The c-Src kinase can be activated by displacement of SH2/SH3 domain through competitive polypeptides or tyrosine dephosphorylation.

Moreover, our studies suggest that activation of SCF by disruption of CUL1's ECTD phenocopy Nedd8-mediated effects (Figs. 3–5), raising a possibility for a role of Nedd8 in regulating the ECTD·ROC1 interaction. Molecular modeling analysis of the ROC1–CUL1–Nedd8 docking complex (22) suggests that Nedd8 conjugation causes conformational changes that disrupt the ECTD·ROC1 interface (data not shown). As noted by previous observations (17), substrate-bound SCF contains CUL1 enriched with the neddylated form, suggesting that substrate ubiquitination and neddylation may be coordinated. In light of these findings, we propose that neddylation/

deneddylation may switch SCF on/off by regulating the interactions between the human CUL1 ECTD and ROC1. While "on duty" with a substrate, SCF must be in an active neddylated form, presumably with the CUL1 ECTD dissociated from ROC1. However, once exited to an "off-duty" state, SCF may become deneddylated through the action of the COP9 signalosome, re-establishing the ECTD·ROC1 interface and turning the E3 into an inactive/restrained form (Fig. 5*D*).

SCF activity is regulated at multiple levels. Recent studies have revealed a role for dimerization of F-box proteins in enhancing catalytic efficiency in ubiquitination (23). In addition, it was observed that the Skp1–Skp2 complex promoted the disassociation of the cullin–CAND1 complex (24), suggesting a role for dynamic interactions among Skp1–F-box protein, cullin, and CAND1 in the assembly of active SCF E3 complexes. Autoinhibition and Nedd8-mediated activation of SCF, as suggested by the current study, provides another mechanism for fine-tuning the SCF activity in accordance with its physiological function.

The human CUL1 ECTD·ROC1 interface is established by contacts between the ROC1 H3 helix and CUL1 H31, as well as S10 (Fig. 2*B*). Aside from binding to the CUL1 ECTD, the ROC1 H3 helix has been predicted to participate in interactions with an E2 as well (13). On this helix, whereas residue K89 interacts with CUL1 Y761 (Fig. 2*B*), W87 is presumed to bind to E2 (13). Mutagenesis experiments revealed that whereas ROC1 K89A activated ubiquitination (Figs. S4 and S5), ROC1 W87A was unable to support polyubiquitin chain assembly by Cdc34 (data not shown). It can be speculated that the CUL1 ECTD·ROC1 H3 interface may present a steric hinderance that restricts Cdc34 loading or may position the ROC1 H3-bound E2 in a manner that is unfavorable to Ub transfer. Disruption of this interaction, resulted from neddylation as discussed above, would stimulate ubiquitination by promoting Cdc34 recruitment and/or by properly aligning the bound E2 in favor of ubiquitination.

Materials and Methods

This study used the following plasmid vectors: pcDNA3.1-Flag-CUL1 (4), pcDNA3.1-HA-βTrCP2 (HOS; ref. 2), and pGEX-4T3/pET-15b-GST-HA-ROC1-Flag-CUL1$^{324-776}$ (6). CUL1 mutants were generated by using a QuikChange Site-Directed Mutagenesis Kit with the mutagenesis primers indicated in *SI Text*. SCF$^{\beta TrCP2}$, ROC1-CUL1$^{324-776}$, or mutant E3 complexes were expressed and affinity-purified by the procedures described in *SI Text*. Several reconstituted *in vitro* ubiquitination assays, including ubiquitination of IκBα, neddylation, and substrate-free polyubiquitin chain assembly, were carried out as described (2, 4, 6–8), with concentrations of the components and reaction conditions as specified in *SI Text*.

Note added in proof. In a recent study (26), Schulman and colleagues have solved the structure of Nedd8-CUL5 C terminus-ROC1/Rbx1, demonstrating that Nedd8 induces a drastic conformational change that abolishes the interaction between ROC1/Rbx1 and CUL5 WH-B domain (equivalent to CUL1 ECTD). This work provides compelling structural evidence supporting for a role of neddylation in regulating the interaction between the CUL1 ECTD and ROC1.

ACKNOWLEDGMENTS. A.S. was supported by the German Research Foundation. This study was supported by Public Health Service Grants GM61051 and CA095634 (to Z.-Q.P.).

1. Petroski MD, Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* 6:9–20.
2. Tan P, *et al.* (1999) Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of IκBα. *Mol Cell* 3:527–533.
3. Ohta T, Michel JJ, Schottelius AJ, Xiong Y (1999) ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Mol Cell* 3:535–541.
4. Wu K, *et al.* (2000) The SCF(HOS/β-TRCP)–ROC1 E3 ubiquitin ligase utilizes two distinct domains within CUL1 for substrate targeting and ubiquitin ligation. *Mol Cell Biol* 20:1382–1393.
5. Chen A, *et al.* (2000) The conserved RING-H2 finger of ROC1 is required for ubiquitin ligation. *J Biol Chem* 275:15432–15439.
6. Wu K, Chen A, Pan Z-Q (2000) Conjugation of Nedd8 to CUL1 enhances the ability of the ROC1–CUL1 complex to promote ubiquitin polymerization. *J Biol Chem* 275:32317–32324.
7. Gazdoiu S, *et al.* (2005) Proximity-induced activation of human Cdc34 through heterologous dimerization. *Proc Natl Acad Sci USA* 102:15053–15058.
8. Gazdoiu S, Yamoah K, Wu K, Pan ZQ (2007) Human cdc34 employs distinct sites to coordinate attachment of ubiquitin to a substrate and assembly of polyubiquitin chains. *Mol Cell Biol* 27:7041–7052.
9. Petroski MD, Deshaies RJ (2005) Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF–Cdc34. *Cell* 123:1107–1120.
10. Furukawa M, Ohta T, Xiong Y (2002) Activation of UBC5 ubiquitin-conjugating enzyme by the RING finger of ROC1 and assembly of active ubiquitin ligases by all cullins. *J Biol Chem* 277:15758–15765.
11. Dias DC, Dolios G, Wang R, Pan Z-Q (2002) CUL7: A DOC domain-containing cullin selectively binds Skp1.Fbx29 to form an SCF-like complex. *Proc Natl Acad Sci USA* 99:16601–16606.
12. Koepp DM, Harper JW, Elledge SJ (1999) How the cyclin became a cyclin: Regulated proteolysis in the cell cycle. *Cell* 97:431–434.
13. Zheng N, *et al.* (2002) Structure of the Cul1–Rbx1–Skp1–F boxSkp2 SCF ubiquitin ligase complex. *Nature* 416:703–709.
14. Lammer D, *et al.* (1998) Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCFCdc4 complex. *Genes Dev* 12:914–926.
15. Furukawa M, Zhang Y, McCarville J, Ohta T, Xiong Y (2000) The CUL1 C-terminal sequence and ROC1 are required for efficient nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1. *Mol Cell Biol* 20:8185–8197.
16. Podust VN, *et al.* (2000) A Nedd8 conjugation pathway is essential for proteolytic targeting of p27Kip1 by ubiquitination. *Proc Natl Acad Sci USA* 97:4579–4584.
17. Read MA, *et al.* (2000) Nedd8 modification of cul-1 activates SCF(β(TrCP))-dependent ubiquitination of IκBα. *Mol Cell Biol* 20:2326–2333.
18. Kawakami T, *et al.* (2001) NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J* 20:4003–4012.
19. Turner GC, Du F, Varshavsky A (2000) Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. *Nature* 405:579–583.
20. Wiesner S, *et al.* (2007) Autoinhibition of the HECT-type ubiquitin ligase Smurf2 through its C2 domain. *Cell* 130:651–662.
21. Roskoski R, Jr (2004) Src protein-tyrosine kinase structure and regulation. *Biochem Biophys Res Commun* 324:1155–1164.
22. Pan Z-Q, Kentsis A, Dias DC, Yamoah K, Wu K (2004) Nedd8 on cullin: Building an expressway to protein destruction. *Oncogene* 23:1985–1997.
23. Tang X, *et al.* (2007) Suprafacial orientation of the SCFCdc4 dimer accommodates multiple geometries for substrate ubiquitination. *Cell* 129:1165–1176.
24. Bornstein G, Ganoth D, Hershko A (2006) Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate. *Proc Natl Acad Sci USA* 103:11515–11520.
25. Jones J, *et al.* (2008) A targeted proteomic analysis of the ubiquitin-like modifier Nedd8 and associated proteins. *J Proteome Res* 7:1274–1287.
26. Duda DM, *et al.* (2008) Structural insights into NEDD8 activation of cullin–RING ligases: Conformational control of conjugation. *Cell*, in press.