The Cullin3 Ubiquitin Ligase Functions as a Nedd8-bound Heterodimer[□]

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Cullins are members of a family of scaffold proteins that assemble multisubunit ubiquitin ligase complexes to confer substrate specificity for the ubiquitination pathway. Cullin3 (Cul3) forms a catalytically inactive BTB-Cul3-Rbx1 (BCR) ubiquitin ligase, which becomes functional upon covalent attachment of the ubiquitin homologue *neural-precursor-cell-expressed and developmentally down regulated 8* (Nedd8) near the C terminus of Cul3. Current models suggest that Nedd8 activates cullin complexes by providing a recognition site for a ubiquitin-conjugating enzyme. Based on the following evidence, we propose that Nedd8 activates the BCR ubiquitin ligase by mediating the dimerization of Cul3. First, Cul3 is found as a neddylated heterodimer bound to a BTB domain-containing protein *in vivo*. Second, the formation of a Cul3 heterodimer is mediated by a Nedd8 molecule, which covalently attaches itself to one Cul3 molecule and binds to the winged-helix B domain at the C terminus of the second Cul3 molecule. Third, complementation experiments revealed that coexpression of two distinct nonfunctional Cul3 mutants can rescue the ubiquitin ligase function of the BCR complex. Likewise, a substrate of the BCR complex binds heterodimeric Cul3, suggesting that the Cul3 complex is active as a dimer. These findings not only provide insight into the architecture of the active BCR complex but also suggest assembly as a regulatory mechanism for activation of all cullin-based ubiquitin ligases.

INTRODUCTION

The conjugation of ubiquitin to a lysine residue of a target protein serves as a signaling mechanism in many biological processes, such as protein trafficking, DNA repair, protein–protein interactions, and proteolysis (Hershko and Ciechanover, 1998; Pines and Lindon, 2005). These ubiquitin linkages are formed by a series of enzymes designated as ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (Ciechanover *et al.*, 1980). A catalytically active ubiquitin ligase directly transfers ubiquitin molecules to the selected protein substrate, where a noncatalytic ubiquitin ligase shuttles the recruited substrate to a ubiquitin-conjugating enzyme (Glickman and Ciechanover, 2002). This latter group of E3 ligases includes cullin-based ubiquitin ligases, in which the cullin acts as a scaffold for the assembly of a multisubunit ubiquitin ligase complex that contains a RING-box protein and a cullin-specific substrate adaptor protein (Ohta *et al.*, 1999; Petroski and Deshaies, 2005). Cullin3 (Cul3) forms a complex called the BTB-Cul3-Rbx1 (BCR) ubiquitin ligase, which controls the degradation of several proteins, including cyclin E, the meiotic spindle-formation factor Mei-1, the transcription factor Nrf2, the Ci/Gli transcription factor, and the Dishevelled protein in the Wnt- β -catenin pathway (Singer *et al.*, 1999; Pintard *et al.*, 2003b; Zhang *et al.*, 2004, 2006; Angers *et al.*, 2006). The BCR com-

plex acquires its substrate specificity through a BTB domain-containing protein bound at the N terminus of Cul3 (Geyer *et al.*, 2003; Xu *et al.*, 2003; Pintard *et al.*, 2004). Meanwhile, a RING-box protein Rbx1 positions its zinc-finger motif near the C terminus of Cul3 to recruit a ubiquitin-conjugating enzyme (Seol *et al.*, 1999). The fully assembled BCR ubiquitin ligase complex is functionally inactive, because all cullins require an attachment of the ubiquitin homologue *neural-precursor-cell-expressed and developmentally down regulated 8* (Nedd8) at a specific lysine residue near its C terminus to activate its ubiquitin ligase function (Hori *et al.*, 1999; Ohh *et al.*, 2002; Pan *et al.*, 2004).

Nedd8 is a highly conserved 81-residue protein that is covalently attached to cullins by a process termed neddylation. The removal of this isopeptide bond between Nedd8 and cullins, or cullin deneddylation, is catalyzed by an isopeptidase enzyme in the COP9 signalosome (CSN) (Lyapina *et al.*, 2001; Zhou *et al.*, 2001; Cope *et al.*, 2002). Many studies have established that neddylation is essential for the activation of cullin-based ubiquitin ligases in several organisms. Nedd8 has been shown to enhance the ability of the ubiquitin-conjugating enzyme Cdc34 to form a ubiquitin chain in a Cul1-dependent manner (Wu *et al.*, 2000, 2002). Nedd8 is also required *in vivo* for normal Cul3 function in *Drosophila* (Ou *et al.*, 2002; Zhu *et al.*, 2005), for early embryonic development in mice (Tateishi *et al.*, 2001), and for the viability of *Caenorhabditis elegans* larvae (Kurz *et al.*, 2002). Furthermore, the addition of the CSN complex to promote Cul1 deneddylation reduces the amount of ubiquitinated substrates *in vitro*, suggesting that Cul1 activity is inhibited when the Nedd8 linkage is removed (Zhou *et al.*, 2003).

Although the significance of Nedd8 in the activation of cullin complexes is firmly established, the mechanism through which Nedd8 functions remains unclear. One study proposed that Nedd8 recruits a ubiquitin-conjugating enzyme to the Cul1

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ubiquitin ligase (Kawakami *et al.*, 2001). However, when this was tested by inactivation of the CSN complex, the percentage of neddylated cullins in cells increased, but the activity of the cullin complex did not respond accordingly. Instead, cullin substrates accumulated, indicating that deneddylation of the cullin complex by CSN is essential for the function of the cullin-based ubiquitin ligases (Schwechheimer and Deng, 2001; Lykke-Andersen *et al.*, 2003). These observations led to a model proposing that a cycle of neddylation and deneddylation is essential to maintain a functional cullin complex (Pintard *et al.*, 2003a).

Here, we show that Nedd8 is required for the formation of a heterodimeric Cul3 complex composed of one neddylated and one unneddylated Cul3. Based on the results from this study, we propose a model in which this Nedd8-bound Cul3 heterodimer is an active form of the BCR ubiquitin ligase complex. This model also supports previous observations regarding the activation of the cullin-based ubiquitin ligase by Nedd8 and the effect of the CSN complex on the activity of cullins.

MATERIALS AND METHODS

Plasmids and Mutagenesis

FLAG-tagged Cul3 or Myc-tagged Cul3 were created by cloning the Cul3 coding region in-frame to the epitope tags in 3XFLAG-myc-24 (Sigma-Aldrich, St. Louis, MO) or the CS2+MT plasmid, respectively. CFP-Cul3 and YFP-Cul3 fusion proteins were created by inserting the full-length Cul3 sequence into pECFP-C1 or pEYFP-C1 plasmids (Clontech, Mountain View, CA). A CFP-actin fusion protein was created by cloning the full-length actin sequence into the pECFP-C1 plasmid. Mutations in the Cul3 protein were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All plasmids were sequenced after mutagenesis. Human SPOP and cyclin E were cloned from a cDNA library into the CS2+MT vector. The HA-Nedd8 plasmid was described previously (Singer *et al.*, 1999; Sheaff *et al.*, 2000). His-tagged Nedd8 was cloned from the HA-Nedd8 plasmid with primers that added a 6xHis sequence to the 5' end of Nedd8.

Cell Culture and Transient Transfection

Human embryonic kidney (HEK)293 cell lines were maintained at 37°C in DMEM with 10% fetal bovine serum (FBS). For transient gene expression, HEK293 cells were transfected using the calcium phosphate precipitation method as described previously (Clurman *et al.*, 1996). A Chinese hamster ovary (CHO) cell line (American Type Culture Collection, Manassas, VA) and a derivative cell line containing a temperature-sensitive mutation (ts41) (from J. Roberts, Fred Hutchinson Cancer Research Center, Seattle, WA) were maintained in Ham's F-12K medium with 10% FBS and 2 mM L-glutamate. For transient gene expression, CHO and ts41 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). CHO cells were maintained at 37°C, whereas ts41 cells were maintained at 32°C.

Fluorescence Resonance Energy Transfer (FRET) Analysis

HEK293, CHO, and ts41 cells were plated on FluoroDish with poly-D-lysine-coated glass bottoms (WPI, Sarasota, FL). HEK293 cells were transiently transfected by calcium phosphate precipitation and incubated at 37°C for 48 h. CHO and ts41 cells were transfected with Lipofectamine 2000 reagent and incubated at 32°C for 48 h for permissive temperature conditions. For nonpermissive temperature conditions, CHO and ts41 cells were incubated at 32°C for 32 h and then shifted to 39°C for 16 h. HEK293, CHO, and ts41 cells were washed twice with phosphate-buffered saline and incubated in HEPES-buffered media before fluorescent microscope analysis. Cells were imaged on Zeiss Axiovert 200M light/fluorescence microscope with a 100× oil objective. The cyan fluorescent protein (CFP) filter uses a 436/20-nm excitation and 480/40-nm emission. The yellow fluorescent protein (YFP) filter uses a 500/20-nm excitation and 535/30-nm emission. The FRET filter uses a 436/20-nm excitation and 535/30-nm emission. Images were obtained sequentially using phase-contrast, CFP, YFP and FRET filters. MetaMorph version 6 (Molecular Devices, Sunnyvale, CA) was used to calculate the net FRET values by using the equation $\text{net FRET} = \text{FRET}_{\text{CFP/YFP}} - (B \times \text{YFP} + A \times \text{CFP})$, where B is FRET/YFP when YFP-Cul3 fusion protein was expressed alone and A is FRET/CFP when CFP-Cul3 fusion protein was expressed alone. The net FRET values were normalized by $\text{net FRET}/\text{FRET} \times 100$ for comparison among different samples.

Immunoblotting and Immunoprecipitation

Cells were harvested in versene (1 mM EDTA in phosphate-buffered saline). Cell lysates were prepared by sonication for 10 s in radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 0.01 M sodium phosphate buffer, pH 7.2, and 2 mM EDTA). For immunoprecipitation assays, the clarified lysates were incubated with primary antibody, followed by protein A-Sepharose beads for 1 h each at 4°C. Beads were spun down and washed twice with RIPA buffer. Protein samples were separated by electrophoresis on 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), and incubated overnight in primary antibodies. Proteins were visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence followed by digital acquisition using an Alpha Innotech (San Leandro, CA) Fluorochem SP system. The following antibodies were used in these experiments: anti-FLAG M2 monoclonal antibody (mAb) (Sigma-Aldrich), anti-Myc sc-789 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-hemagglutinin (HA) HA.11 mAb (Covance, Princeton, NJ), anti-Nedd8 antibody (Alexis Laboratories, San Diego, CA), anti-cyclin E HE111 (Santa Cruz Biotechnology), and anti-Cul3 antibody, which has been described previously (Singer *et al.*, 1999).

Purification of the FLAG-tagged Cul3 Dimer with His-tagged Nedd8

HEK293 cells were transiently transfected with vectors encoding FLAG-tagged Cul3 and His-tagged Nedd8. Cells were harvested, and lysates were prepared by sonication in RIPA buffer. Lysates were centrifuged at $18,000 \times g$ at 4°C for 5 min to pellet cell debris, and the FLAG-tagged Cul3 dimer with His-tagged Nedd8 was purified using BD Talon Metal Affinity Resin (BD Biosciences, San Jose, CA). The purified Cul3 dimer was collected and concentrated with Centrplus centrifugal filter devices (Millipore, Billerica, MA) before loading onto the size exclusion column.

Purification of the His-tagged Cul3 Monomer

BL21-Gold (DE3) pLysS *Escherichia coli* strain transformed with the pET16Cul3 vector were grown in 300 ml of LB media supplemented with 100 µg/ml ampicillin to an OD₆₀₀ of 0.6. The expression of His-tagged Cul3 protein was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) (final concentration of 1 mM) for 3 h before harvesting. Cells were harvested and His-tagged Cul3 protein was extracted using Bugbuster protein extraction reagent (Novagen, San Diego, CA). Soluble His-tagged Cul3 protein was purified using BD Talon Metal Affinity Resin (BD Biosciences), and the eluted protein was collected for size exclusion chromatography.

Size Exclusion Chromatography

The purified Cul3 complex was applied to a Superdex 200 column equilibrated with 50 mM phosphate buffer containing 0.15 M NaCl. The eluted protein was collected in 0.5-ml fractions, precipitated with acetone, and dissolved in water for further analysis by immunoblotting.

Cyclin E In Vivo Ubiquitination Assay

HEK293 cells were transiently transfected with Myc-tagged cyclin E, HA-tagged ubiquitin, and CS2+ Cul3. Cells were harvested, sonicated in RIPA buffer, and immunoprecipitated for ubiquitinated Myc-tagged cyclin E.

Yeast Two-Hybrid and β-Galactosidase Assays

Saccharomyces cerevisiae EGY48 cells containing pGilda-Nedd8 and p8opLacZ were transformed with either pB42AD-Cul3 or pB42AD-Cul3K712R plasmids and grown on – UTH minimum SD media with 2% dextrose. For the negative control, pGilda-Nedd8 plasmids were replaced with pLexA-lamin. The interactions between proteins expressed from pGilda-Nedd8 and pB42AD-Cul3 or pB42AD-Cul3K712R were quantified by measuring the β-galactosidase activity by using chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate. To calculate the final β-galactosidase value, the average β-galactosidase value obtained from the negative control strain was subtracted from the average β-galactosidase values obtained from the yeast strains containing pGilda-Nedd8 plasmids.

RESULTS

Cul3–Cul3 Intermolecular Interactions Are Detected In Vivo

The COP-1 ubiquitin ligase has been established previously to function as a homodimer (Torii *et al.*, 1998); therefore, we investigated whether other ubiquitin ligases, such as the Cul3-based ubiquitin ligase, can also form functional dimers in vivo. To test this hypothesis, Cul3 fusion proteins CFP-Cul3 and YFP-Cul3 were coexpressed in HEK293 cells for FRET analysis. Cells were stimulated with the CFP excita-

Figure 1. Cul3-Cul3 intermolecular interactions are detected in vivo. (A) HEK293 cells expressing CFP-Cul3 (a-e), YFP-Cul3 (f-j), and CFP-Cul3 with YFP-Cul3 (k-o) were imaged for quantitative FRET analysis. Phase contrast (a, f, and k), CFP (b, g, and l), YFP (c, h, and m), and FRET fluorescence images (d, i, and n) were acquired and net FRET values (e, j, and o) were determined using the MetaMorph application. (B) Normalized net FRET values of full-length Cul3 in HEK293 cells expressing CFP-Cul3 (left), YFP-Cul3 (middle), and CFP-Cul3 with YFP-Cul3 (right). (C) HEK293 cells were transfected with vectors expressing FLAG-tagged and Myc-tagged Cul3 or a non-BTB binding mutant (L52AE55A) as indicated. Lysates were prepared, checked for protein expression (bottom), and immunoprecipitated with anti-Myc antibody. The precipitates were separated by SDS-PAGE and immunoblotted with anti-FLAG antibody (top). The upper band of the Cul3 doublet represents the Nedd8-conjugated form of Cul3. (D) FLAG-tagged Cul3 and Myc-tagged Cul3, Cul3K712R mutant, Cul3patch A mutant (A705D A706D I707E V708E), Cul3patch B mutant (P738E V739E V740D I741D), Cul3K712R patch B mutant, or Cul3patch A patch B mutant were expressed in HEK293 cells (middle). Lysates were prepared, and proteins were subjected to immunoprecipitation with anti-Myc antibody and immunoblotting with anti-FLAG antibody (top). Relative locations of the Cul3 mutants are illustrated by sequence alignment of human Cul1, Cul2, and Cul3 along with the predicted secondary structure of the WH-B domain (bottom).

tion wavelength (436 nm), and the YFP emission wavelength (535 nm) was detected (Figure 1A, d, i, and n). After subtracting FRET emissions from the negative controls, 44% of cells expressing both CFP-Cul3 and YFP-Cul3 showed high net FRET values, indicating that CFP-Cul3 binds to YFP-Cul3 (Figure 1A, o). The intensity of net FRET values from Figure 1A were quantified, normalized against the total FRET emission to control for differential protein expression in each cell, and compared with net FRET values from cells that expressed either CFP-Cul3 or YFP-Cul3 alone (Figure 1B). No net FRET with YFP-Cul3 was seen when CFP-Cul3 was substituted with CFP-actin (Supplemental Figure 1).

BTB Domain-containing Proteins Are Not Required for Cul3-Cul3 Binding

We performed coimmunoprecipitation assays to verify the Cul3-Cul3 binding interaction that was observed using

FRET analysis. FLAG-tagged Cul3 and Myc-tagged Cul3 were coexpressed in HEK293 cells and tested for protein expression (Figure 1C, bottom two lanes). Myc-tagged Cul3 was able to pull down FLAG-tagged Cul3 (Figure 1C, lane 3), indicating potential interactions between two or more Cul3 molecules. Because BTB domain-containing proteins can form homodimers (Robinson and Cooley, 1997), it has been proposed that the dimerization of BTB domain-containing proteins bound to each Cul3 partner could promote Cul3-Cul3 interactions (Stogios *et al.*, 2005). To test this hypothesis, a Cul3 mutant (Cul3L52AE55A) that cannot bind any BTB domain-containing protein (Zheng *et al.*, 2002; Xu *et al.*, 2003) (Figure 4B, lane 3) was selected for immunoprecipitation assays. If Cul3 dimerization occurs through the BTB domain-containing protein, the Cul3L52AE55A mutant would not be able to sustain the Cul3-Cul3 interactions. We observed that the Myc-tagged Cul3L52AE55A pulled down

FLAG-tagged Cul3L52AE55A with the same efficiency as the wild-type Cul3 pair (Figure 1C, lane 4), suggesting that a BTB domain-containing protein is not required for the association of the Cul3–Cul3 complex.

The Cul3–Cul3 Association Is Mediated by a Winged-Helix B (WH-B) Domain near the C-Terminus of Cul3

Based on observations that a ubiquitin molecule can mediate protein dimerization (Mueller and Feigon, 2002), we investigated whether the conjugation of Nedd8 to the C-terminal domain of Cul3 participates in these Cul3–Cul3 interactions. To inhibit Nedd8 conjugation, the neddylation site lysine 712 near the C terminus of Cul3 was mutated to arginine, creating the Cul3K712R mutant (Wu *et al.*, 2000; Zheng *et al.*, 2002). Myc-tagged Cul3K712R was able to pull down FLAG-tagged Cul3K712R with a slight decrease in efficiency compared with the wild-type pair (Figure 1D, lanes 3 and 4), showing that covalent attachment of Nedd8 is not necessary for these Cul3–Cul3 interactions. Because lysine 712 lies within the WH-B domain that contains a stretch of hydrophobic residues (Zheng *et al.*, 2002), we mutated these residues in helix 1 (patch A mutant) and helix 3 (patch B mutant) (Figure 1D, bottom). All Cul3 mutants were able to bind Rbx1 and BTB domain-containing proteins (data not shown), suggesting that the mutation did not disrupt protein folding. Immunoprecipitation revealed that either the patch A or the patch B mutants alone reduce Cul3–Cul3 binding; however, when both mutations were made in the same molecule, Cul3–Cul3 interactions were completely disrupted (Figure 1D, lanes 5–8).

The Availability of a Nedd8 Molecule Is Essential for Cul3–Cul3 Interactions

Because mutations in the WH-B domain inhibited Cul3–Cul3 interactions, we decided to further investigate the *in vivo* role of Nedd8 in this particular example of Cul3–Cul3 binding. FRET experiments were performed in a Chinese hamster ovary cell line with a temperature-sensitive mutation in the neddylation pathway (ts41 cells) to determine whether Nedd8 is required to facilitate these Cul3–Cul3 interactions. If the presence of Nedd8 is essential for the dimerization of Cul3, Cul3–Cul3 interactions should be disrupted at the nonpermissive temperature at which the Nedd8 conjugation pathway becomes nonfunctional and thus drastically reduces the amount of Nedd8 localized to Cul3 (Handeli and Weintraub, 1992). However, if the interaction between Nedd8 and Cul3 is not required, Cul3–Cul3 binding should persist even at nonpermissive temperature. Ts41 cells expressing CFP–Cul3 and YFP–Cul3 were incubated at either permissive temperature (32°C) or nonpermissive temperature (39°C) before the FRET analysis. At permissive temperature (32°C), Cul3 fusion proteins yielded a high net FRET value, indicating strong Cul3–Cul3 interactions (Figure 2A, lanes a–e). However, the net FRET values decreased at nonpermissive temperature (39°C) (Figure 2A, lanes f–j), suggesting that Cul3–Cul3 interactions were minimized in the absence of a Nedd8 molecule. To confirm that Cul3 neddylation is inhibited at nonpermissive temperature (39°C), immunoprecipitation followed by immunoblotting of endogenous Cul3 protein was performed on ts41 cell lysates. Although the population of unneddylated Cul3 remained the same at both temperatures, the population of neddylated Cul3 was reduced drastically at nonpermissive temperature (39°C), confirming that the reduction of Cul3–Cul3 interaction was a direct result of the disrupted neddylation pathway (Figure 2B). As an experimental control,

Figure 2. The neddylation pathway is essential for Cul3–Cul3 interactions. (A) Ts41 cells expressing CFP–Cul3 and YFP–Cul3 proteins were used for quantitative FRET imaging. Cells were incubated at permissive temperature (32°C) (a–e) and nonpermissive temperature (39°C) (f–j) for 16 h before the FRET measurement. The net FRET values were calculated using MetaMorph (e and j). (B) Ts41 cell lysates at permissive (32°C) and nonpermissive (39°C) temperature were immunoprecipitated with anti-Cul3 antibody. The precipitates were separated by SDS-PAGE and immunoblotted with anti-Cul3 antibody. (C) The net FRET values from ts41 cells and CHO cells expressing CFP–Cul3 and YFP–Cul3 were normalized, and data from permissive (32°C) (blue) and nonpermissive (39°C) (red) temperatures were compared.

CFP–Cul3 and YFP–Cul3 were expressed in CHO cells and subjected to the same conditions as the ts41 cells. Net FRET values detected from CHO cells are similar at 32 and 39°C (Figure 2C), confirming that the decrease in Cul3–Cul3 interactions was due to the nonfunctional Nedd8 pathway and not other temperature-dependent factors. The results from this FRET analysis support our hypothesis that the presence of Nedd8 is essential for Cul3–Cul3 interactions.

Nedd8 Mediates the Formation of a Cul3 Heterodimer Composed of One Neddylated and One Unneddylated Cul3

Ubiquitin-mediated protein–protein interactions depend on hydrophobic binding between the protein partners and the hydrophobic surfaces of the ubiquitin molecule; therefore, we investigated the type of interactions that Nedd8 requires for participating in Cul3–Cul3 binding. HA-tagged Nedd8 in HEK293 cell lysates was immunoprecipitated with anti-HA antibody, immunoblotted, and probed for endogenous Cul3. A Cul3 doublet containing both the Nedd8-conjugated and the unconjugated Cul3 bands of similar intensity was observed (Figure 3A, left, lane 2). This ratio differed from the proportion of the total Cul3 population in

Figure 3. Nedd8 mediates the formation of Cul3 heterodimers by interacting with the winged-helix B domain near the C termini of Cul3. (A) HEK293 cells were transfected with HA-tagged Nedd8 (left) or harvested without transfection (right). Cell lysates were immunoprecipitated with anti-Cul3 (left, lanes 1 and 3; right, lane 1), anti-HA (left, lanes 2 and 4), or anti-Nedd8 (right, lane 2) antibodies. The precipitates were separated by SDS-PAGE and immunoblotted with anti-Cul3 (left, lanes 1 and 2; right) and anti-HA (left, lanes 3 and 4) antibodies, respectively. The intensity of Cul3 bands was quantified, and the highest value from each lane was normalized to 100% (bottom). (B) HEK293 cells expressing FLAG-tagged Cul3 and His-tagged Nedd8 were harvested. Cell lysates were prepared and the FLAG-tagged Cul3 complex was purified by precipitating via His-tagged Nedd8. Eluants were loaded onto a gel filtration column, and fractions were collected, acetone precipitated, and immunoblotted with anti-FLAG antibody (top). His-tagged Cul3 protein encoded on the pET16Cul3 vector was expressed via IPTG induction in BL21Gold (DE3) pLysS *E. coli*. Cells were lysed and the His-tagged Cul3 monomer was purified. The Cul3 monomer was loaded onto a gel filtration column and fractions were collected and immunoblotted with anti-Cul3 antibody (bottom). (C) HEK293 cells were transfected with vectors expressing FLAG-tagged Cul3, FLAG-tagged Cul3 mutants, and HA-tagged Nedd8 as indicated. Proteins were immunoblotted with anti-FLAG antibody (bottom) to verify expression. Cell lysates were subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-FLAG antibody (top). (D) Proposed model for the formation of Cul3 heterodimers. The Cul3 dimer is held together by Nedd8 through both an isopeptide linkage and hydrophobic interactions to the Nedd8 surface.

cell lysates, in which the upper protein band (Nedd8-conjugated) was less than half as intense as the lower protein band (Nedd8-unconjugated) (Figure 3A, left, lane 1). The same immunoblot was stripped and reprobed with anti-HA antibody to verify that the upper band of the Cul3 doublet is a neddylated form of Cul3 (Figure 3A, left, lanes 3 and 4). To confirm that the Nedd8-bound Cul3 complex occurs endogenously, Nedd8 from untransfected HEK293 cells was immunoprecipitated with anti-Nedd8 antibody, immunoblotted, and probed for Cul3, revealing nearly equivalent precipitation of neddylated and unneddylated Cul3 (Figure 3A, right, lane 2). These results demonstrate two significant findings. First, it is expected that Nedd8 immunoprecipitation would pull down the Nedd8-conjugated Cul3 based on previous observations that Nedd8 can covalently attach to the lysine residue near the C terminus of cullins (Osaka *et al.*, 1998; Hori *et al.*, 1999; Wada *et al.*, 1999). However, our

observation that Nedd8 immunoprecipitated unconjugated Cul3 was unexpected and revealed a novel noncovalent interaction between Nedd8 and the Cul3 protein. Second, the Nedd8-bound Cul3 complex contains approximately equal amounts of neddylated and unneddylated Cul3, which is different from the proportion of neddylated and unneddylated Cul3 found in cell lysates.

To further investigate the observed noncovalent interaction between Nedd8 and Cul3, the binding between the two proteins was investigated in a two-hybrid assay. The relative strength of the interactions was determined using a β -galactosidase liquid culture assay with CPRG as a substrate. After subtraction of data from controls, we observed that the Cul3K712R mutant was able to bind to Nedd8 in cells although with reduced efficiency (89.7% for wild type and 30.3% for Cul3K712R) (Table 1). Together, these data confirm a second, noncovalent interaction between Nedd8 and Cul3 in both yeast and mammalian cells.

Table 1. Relative binding between Nedd8 and Cul3 or a Cul3K712R mutant in *S. cerevisiae*

LexA fusion vector	Activation domain fusion vector	Lac Z reporter vector	β -Galactosidase activity ^a (%)
pGilda-Nedd8	pB42AD-Cul3	p8opLacZ	89.7 + 8.3
pGilda-Nedd8	pB42AD-Cul3K712R	p8opLacZ	30.3 + 8.7

^a The relative binding between Nedd8 and Cul3 was measured using a β -galactosidase liquid culture assay with CPRG as the substrate.

To determine the stoichiometry of the multimeric Cul3 complex, purified complexes were analyzed using a gel filtration column. FLAG-tagged Cul3 was pulled down by His-tagged Nedd8 expressed in HEK293 cells and used to determine the size of the Nedd8-bound Cul3 complex. Purified Cul3 monomers from *E. coli* were used as a control in this experiment. Because Nedd8 and the neddylation pathway do not exist in *E. coli*, the possibility of Nedd8-mediated Cul3 heterodimers in the control sample is eliminated. His-tagged Cul3 from *E. coli* was purified to obtain Cul3 monomers, analyzed by gel filtration, immunoblotted, and probed for Cul3 with anti-Cul3 antibody (Figure 3B, bottom). Cul3 purified from *E. coli* eluted from the column close to the predicted molecular mass of the Cul3 monomer (90 kDa). In contrast, the Nedd8-bound Cul3 complex was eluted at ~216 kDa, which is approximately the same size as the predicted mammalian Cul3 dimer consisted of a single Nedd8 molecule, two Rbx1 proteins, and two Cul3 monomers (Figure 3B, top). Therefore, these Cul3 complexes are dimers and do not represent a higher oligomeric state complex. These experimental observations led to the conclusion that Nedd8 mediates the formation of Cul3 heterodimers composed of one neddylation and one unneddylation Cul3.

Based on the proximity of the Rbx1 binding site to the site of Nedd8 attachment in the C-terminal domain of Cul3, Rbx1 was also tested for its ability to mediate the formation of Cul3 heterodimer. An immunoprecipitation of Rbx1 brought down neddylation and unneddylation Cul3 in the same ratio as that found in cell lysates (Supplemental Figure 2) and not the one-to-one ratio observed in the Nedd8 immunoprecipitation assays, suggesting that Rbx1 does not facilitate the formation of this Cul3 dimer.

Both an Isopeptide Linkage and Hydrophobic Binding between Nedd8 and the WH-B Domains of Cul3 Contribute to the Nedd8-mediated Cul3 Dimerization

Previous observations stating that the WH-B domain is essential for Cul3–Cul3 interactions and that Nedd8 is required for Cul3–Cul3 binding have led us to investigate whether the WH-B domain interacts directly with the Nedd8 molecule. To analyze the potential role of this domain in the formation of the Cul3 dimer, we immunoprecipitated HA-tagged Nedd8 and probed with anti-FLAG antibody for binding to FLAG-tagged Cul3 variants with mutations in the WH-B domain. As a negative control, cell lysates expressing FLAG-tagged Cul3 alone were immunoprecipitated with anti-HA antibody, proving that FLAG-tagged Cul3 was not pulled down nonspecifically by this immunoprecipitation (Figure 3C, lane 1). When coexpressed with HA-tagged Nedd8, however, wild-type FLAG-tagged Cul3 and a FLAG-tagged Cul3L52AE55A mutant were pulled down as doublets with equal band intensities (Figure 3C, lanes 2 and 3), confirming that a BTB domain-containing protein does not mediate the formation of Cul3 dimers. If an isopeptide bond between Nedd8 and Cul3 is the only interaction that holds the two molecules together, HA-tagged Nedd8 would not pull down a FLAG-tagged Cul3K712R mutant because it lacks the neddylation site (Supplemental Figure 3, lane 2). However, this study showed that HA-tagged Nedd8 was bound to the unneddylation Cul3K712R mutant, which further supports our observation that the second type of Nedd8–Cul3 interaction is noncovalent (Figure 3C, lane 4). An immunoprecipitation of the Cul3 patch B mutant via Nedd8 shows a slightly greater amount of neddylation Cul3 than unneddylation Cul3 (Figure 3C, lane 6), suggesting that hydrophobic residues in the WH-B domain of Cul3 participate in the dimerization. When a double mutant was made by combining K712R with the patch B mutations,

only the unneddylation form of the Cul3 mutant was bound to Nedd8 (Figure 3C, lane 7). A similar result was also found in the Cul3 patch A mutant, which has a partially disrupted hydrophobic surface and cannot be neddylation (Figure 3C, lane 5). When both patch mutants were present on the same molecule, HA-tagged Nedd8 did not pull down any FLAG-tagged Cul3 mutants, indicating that both the neddylation site and the hydrophobic region in the WH-B domain are essential for the Nedd8–Cul3 interactions (Figure 3C, lane 8; Supplemental Figure 3, lane 3).

Having demonstrated that Nedd8 mediates Cul3 dimerization, we suggest that two interactions contribute to the dimerization of Cul3: one Cul3 molecule interacts with Nedd8 via a combination of hydrophobic interactions and an isopeptide linkage between Nedd8 and lysine 712. The second Cul3 molecule uses hydrophobic binding between its WH-B domain and the surface of the Nedd8 protein (Figure 3D).

The Cul3 Heterodimer Can Bind a BTB Domain-containing Protein

We suggest that the Cul3 heterodimer is an active form of the Cul3-based ubiquitin ligase complex. Based on the current model of an active Cul3 complex, Cul3 requires a BTB domain-containing protein to provide substrate specificity. A BTB domain-containing protein, SPOP, which is involved in X-chromosome inactivation (Hernandez-Munoz *et al.*, 2005; Kwon *et al.*, 2006; Zhang *et al.*, 2006), was tested for binding to the Cul3 dimer. We observed that HA-tagged Nedd8 did not bind Myc-tagged SPOP alone (Figure 4A, top, lane 2); however, HA-tagged Nedd8 was able to pull down Myc-tagged SPOP in the presence of wild-type Cul3 (Figure 4A, top, lane 3) but not in the presence of the Cul3L52AE55A mutant (Figure 4A, top, lane 4). Nedd8 interactions with a Cul3 dimer and a BTB domain-containing protein suggest that a BTB domain-containing protein can be a member of the heterodimeric Cul3 complex.

Figure 4. The Cul3 heterodimer can bind a BTB domain-containing protein. (A) HEK293 cells were transfected with vectors encoding HA-tagged Nedd8, Myc-tagged SPOP, FLAG-tagged Cul3, and a non-BTB-binding Cul3 mutant (L52AE55A) as indicated. Protein expression was shown by immunoblotting with anti-FLAG or anti-Myc antibodies (bottom two panels). Cell lysates were subjected to immunoprecipitation with anti-FLAG (middle) or anti-HA antibodies (top) followed by immunoblotting with anti-Myc antibody. (B) HEK293 cells were transfected with vectors encoding Myc-tagged SPOP, His-tagged Cul3, FLAG-tagged Cul3, and a non-BTB binding Cul3 mutant (L52AE55A). Protein expression was shown by immunoblotting with anti-FLAG, anti-His, and anti-Myc antibodies (bottom three panels). Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody and immunoblotting with anti-Myc antibody (top).

To confirm the presence of a BTB domain-containing protein in the Cul3 dimer, we used the Cul3L52AE55A mutant that cannot bind a BTB domain-containing protein (Xu *et al.*, 2002; Zheng *et al.*, 2002). However, a Cul3 dimer consisting of a wild-type Cul3 and a Cul3L52AE55A mutant should enable the Cul3 mutant to bind a BTB domain-containing protein through its Cul3 partner. If a BTB domain-containing protein does not bind to a Cul3 dimer, a Cul3L52AE55A mutant would not form a complex with a BTB domain-containing protein regardless of the expression of wild-type Cul3.

To test this hypothesis, either a FLAG-tagged wild-type Cul3 or a FLAG-tagged Cul3L52AE55A mutant, Myc-tagged SPOP, and His-tagged wild-type Cul3 were coexpressed in HEK293 cells and subjected to immunoprecipitation. Myc-tagged SPOP was brought down by FLAG-tagged wild-type Cul3 but not by a FLAG-tagged Cul3L52AE55A mutant (Figure 4B, top, lanes 2 and 3). Immunoprecipitation with anti-FLAG antibody could not coimmunoprecipitate Myc-tagged SPOP in cells that expressed only Myc-tagged SPOP and His-tagged Cul3 (Figure 4B, top, lane 1). However, immunoprecipitation with anti-FLAG antibody coimmunoprecipitated Myc-tagged SPOP when FLAG-tagged Cul3L52AE55A, Myc-tagged SPOP, and His-tagged wild-type Cul3 were coexpressed (Figure 4B, lane 4), suggesting that the FLAG-tagged Cul3L52AE55A formed a dimer with the His-tagged wild-type Cul3 that is bound to Myc-tagged SPOP. These results confirm that a Cul3 heterodimer can bind a BTB domain-containing protein.

A Cul3 Substrate Pulls Down a Heterodimeric Cul3 Complex

The proposed model that the Cul3 heterodimer is an active form of the Cul3-based ubiquitin ligase complex is supported by the ability of the Cul3 heterodimer to bind BTB domain-containing proteins. However, the role of a cullin-based ubiquitin ligase complex is to provide substrate specificity for the ubiquitination pathway by binding to its substrates and recruiting the E2 ubiquitin-conjugating enzyme. Therefore, the presence of protein substrates bound to the ubiquitin ligase complex is key to identifying active ubiquitin ligases. If the active Cul3 complex is a neddylated Cul3 monomer bound to a BTB domain-containing protein, immunoprecipitation of Cul3 substrates should only coimmunoprecipitate neddylated Cul3. However, if the Cul3 heterodimer is the active complex, Cul3 substrates would coimmunoprecipitate the Cul3 dimer with equal amounts of neddylated and unneddylated Cul3. We selected a known Cul3 substrate, cyclin E, to determine whether the Cul3 heterodimer is the active Cul3 complex. Immunoprecipitation of cyclin E brought down FLAG-tagged Cul3 as a doublet similar to the Cul3 doublet from HA-Nedd8 immunoprecipitation assays (Figure 5, right lane). In contrast, immunoprecipitation and immunoblotting for FLAG-tagged Cul3 brought down the same proportion of Cul3 as that found in cell lysates (Figure 5, left lane). The ability of the Cul3 heterodimer to bind its substrate supports the hypothesis that the Cul3 heterodimer is an active form of the Cul3-based ubiquitin ligase complex.

Coexpression of Two Nonfunctional Cul3 Mutants Rescues Its Ubiquitin Ligase Function

Although we demonstrated that the Cul3 heterodimer can bind a BTB domain-containing protein and its substrate, a functional assay is required to prove that Cul3 is active in its heterodimeric form. We have previously established that cyclin E is a substrate of the Cul3-based ubiquitin ligase complex and that coexpression of Cul3 with cyclin E in-

Figure 5. A Cul3 substrate, cyclin E, binds Cul3 in a heterodimeric form. FLAG-tagged Cul3 expressed in HEK293 cells was immunoblotted with anti-FLAG antibody (bottom). Cell lysates were subjected to immunoprecipitation with anti-FLAG (lane 1) or anti-cyclin E antibodies (lane 2) followed by immunoblotting with anti-FLAG antibody (top).

creases the amount of ubiquitinated cyclin E in HEK293 cells (Singer *et al.*, 1999). Similar to the binding assay for a BTB domain-containing protein in Figure 4B, we developed an *in vivo* rescue assay for cyclin E ubiquitination using two different Cul3 mutants that cannot individually ubiquitinate cyclin E. If Cul3 functions only as a monomer, cyclin E bound at the C terminus of Cul3 would only be ubiquitinated by an E2 bound at the C terminus of the same Cul3 molecule. However, if Cul3 can also function as a dimer, a substrate bound to the N terminus of one Cul3 molecule could potentially be ubiquitinated by the E2 bound at the C terminus of the second Cul3 molecule, leading to increased cyclin E ubiquitination when two mutants are coexpressed.

Myc-tagged cyclin E and HA-tagged ubiquitin were coexpressed with either wild-type Cul3 or Cul3 mutants. Immunoblotting was performed to determine relative expression levels of Cul3 proteins (Figure 6A, bottom). Immunoprecipitation with anti-Myc antibody followed by immunoblotting that probes for Myc-tagged cyclin E revealed that coexpression of wild-type Cul3 results in the same high-molecular-weight cyclin E smear that could be induced by the addition of proteasome inhibitor MG132 (Figure 6A, top and middle, lanes 2 and 3). For the rescue assay, two Cul3 deletion mutants were chosen: a Cul3 mutant with a deletion of the first 23 residues of the N terminus (Cul3ΔN) and a Cul3 mutant with a stop codon at residue 734 (Cul3stop734). Although a significant C-terminal portion has been deleted, Cul3 stop734 mutant can form a dimer by using an intact helix-1 in the WH-B domain. Individually, these Cul3 mutants did not induce cyclin E ubiquitination (Figure 6A, lanes 4 and 5). However, cyclin E ubiquitination was rescued when the mutants were coexpressed, indicating that the Cul3 dimer is a functional form of the Cul3-based ubiquitin ligase complex. To confirm that the high-molecular-weight bands were polyubiquitinated cyclin E, the blot was stripped and reprobed with anti-HA antibody (Figure 6B, top). Furthermore, the same lysates were subjected to immunoprecipitation with HA-ubiquitin and probed for cyclin E. The presence of a single band of cyclin E rather than a smear indicates that the most prominent ubiquitinated form of cyclin E is the monoubiquitinated form. Although the endogenous cellular machinery can catalyze the ubiquitination of cyclin E (Figure 6B, middle, lane 1), overexpression of Cul3 protein increases the level of the ubiquitinated cyclin E close to that obtained by an addition of proteasome inhibitor, MG132 (Figure 6B, middle, lanes 2 and 3). The Cul3ΔN and Cul3stop734 mutants yielded the two lowest amount of ubiquitinated cyclin E in this experiment, indicating that these mutants may function as dominant negative and prevent the cyclin E from being ubiquitinated by the endogenous proteins (Figure 6B, middle, lanes 4 and 5). When the two mutants were coexpressed, the Cul3 ubiquitina-

Figure 6. The Cul3 heterodimer is an active form of the Cul3-based ubiquitin ligase complex. (A) HEK293 cells were transiently transfected with vectors encoding untagged Cul3, N-terminally truncated Cul3 mutant (ΔN), C-terminally truncated Cul3 mutant (stop734), Myc-tagged cyclin E, and HA-tagged ubiquitin as indicated. The proteasome inhibitor MG132 was added to the transfected cells in lane 2 18 h before harvesting. Total Cul3 protein expression from the lysates is shown (bottom). Relative levels of cyclin E ubiquitination were determined by immunoprecipitation and immunoblotting with anti-Myc antibody (top and middle). (B) The immunoblot from A was then stripped and reprobed with anti-HA antibody (top). The same lysates were also immunoprecipitated with anti-HA antibody and immunoblotted with anti-Myc antibody to determine the relative levels of ubiquitinated cyclin E in each lane (middle). Arrow denotes the position of nonubiquitinated cyclin E. The amount of ubiquitinated cyclin E was quantified relative to the amount in lane 2, which was normalized to 100% (bottom).

tion activity was rescued (Figure 6B, middle, lane 6), which is similar to the results seen in Figure 6A. Quantitative measurements for the intensity of ubiquitinated cyclin E bands also support this observation (Figure 6B, bottom).

DISCUSSION

In this study, we investigated the architecture and function of an active Cul3-based ubiquitin ligase complex. First, we demonstrated that the WH-B domain near the C terminus of Cul3 mediates Cul3–Cul3 interactions. Second, we identified that Nedd8 facilitates this Cul3–Cul3 binding by forming an isopeptide bond to one Cul3 molecule and interacting with hydrophobic residues in the WH-B domain of the other Cul3 molecule, creating a Cul3 heterodimer. Third, a Cul3 substrate and a BTB domain-containing protein bind to the Cul3 heterodimer, suggesting that this Cul3 complex can select protein substrates for the ubiquitination pathway. Last, we showed that the Cul3 heterodimer is an active ubiquitin ligase. We performed a complementation assay in which the ubiquitin ligase function of Cul3 can be rescued by the coexpression of two distinct Cul3 mutants that are not functional when expressed alone. Therefore, we propose that Nedd8 activates the Cul3-based ubiquitin ligase by facilitating the formation of a Cul3 heterodimer.

The WH-B Domain of Cul3 Forms a Novel Hydrophobic Interaction with a Nedd8 Molecule

Previous observations revealed that an active BCR ubiquitin ligase requires the covalent attachment of a Nedd8 molecule to a Cul3 monomer (Wu *et al.*, 2000; Ou *et al.*, 2002), leading to a prediction that an immunoprecipitation of Nedd8 would bring down only neddylated Cul3. However, we performed a Nedd8 immunoprecipitation that pulled down an endogenous Cul3 dimer composed of one neddylated

and one unneddylated Cul3 (Figure 3A), suggesting that endogenous Cul3 also forms a second type of Cul3–Nedd8 interaction in addition to an isopeptide bond. We searched for a three helical bundle containing a stretch of hydrophobic residues within the Cul3 protein by comparing the crystal structure of Cul1 and sequence alignments among Cul1, Cul2, and Cul3 (Zheng *et al.*, 2002) (Figure 1D, bottom). A WH-B domain near the C terminus of Cul3 was tested for its ability to form hydrophobic interactions with Nedd8 because it contains a Nedd8 conjugation site and stretches of hydrophobic residues within its α -helices. Mutational analysis indicated that Nedd8 binds to the hydrophobic residues in the WH-B domain of Cul3 (Figure 3C), confirming that Nedd8 mediates the dimerization of the Cul3 complex by forming two different interactions with Cul3 proteins: an isopeptide linkage and hydrophobic interactions (Figure 3D).

Hydrophobic Interactions between Cul3 and Nedd8 Are Sufficient to Mediate the Formation of the Cul3 Dimer

It has been established that a single ubiquitin molecule can mediate protein–protein interactions by binding to a domain containing a three helical bundle. Examples include a ubiquitin-associated domain and a coupling of ubiquitin conjugation to ER degradation domain (Wilkinson *et al.*, 2001; Kang *et al.*, 2003; Prag *et al.*, 2003; Shih *et al.*, 2003). The hydrophobic surface of the ubiquitin molecule binds to a stretch of hydrophobic residues within these domains and holds the two proteins together as a dimer. We reasoned that Nedd8 could mediate Cul3 dimerization in a similar manner because Nedd8 is a ubiquitin-like protein with 55% sequence identity and 80% homology to ubiquitin (Kamitani *et al.*, 1998; Wu *et al.*, 2000; Parry and Estelle, 2004). Our hypothesis is further supported by FRET analysis in ts41 cells, which demonstrated that Nedd8 is essential for the efficient formation of Cul3 dimers in vivo (Figure 2). Furthermore,

Figure 7. Proposed models for an active Cul3 heterodimer. A cartoon representation of the Cul3 heterodimer is illustrated based on the predicted Cul3 structure and the published Nedd8 crystal structure, showing that the formation of the Cul3 heterodimer positions the E2 binding site of one Cul3 molecule (green) in proximity to the binding site of a BTB domain-containing protein of the other Cul3 molecule (red). A single Nedd8 (yellow) is held between the two Cul3 molecules by a pair of WH-B domains. (A) The Cul3 dimer in a head-to-head arrangement. (B) The Cul3 dimer in a head-to-tail arrangement.

we discovered that hydrophobic binding between Nedd8 and Cul3 is the primary interaction that holds the two Cul3 molecules together in a dimer as shown in two separate experiments in this study. First, Nedd8 molecules were able to pull down the Cul3K712R mutants in the absence of Nedd8 conjugations, indicating that Cul3 is capable of binding Nedd8 molecule by using only hydrophobic interactions (Figure 3C). Second, the reciprocal immunoprecipitation assays confirm the binding interactions between two Cul3K712R molecules (Figure 1D), indicating that hydrophobic interactions alone are sufficient to mediate the formation of the Cul3 dimer.

Role of Nedd8 Conjugation In Vivo

Although Nedd8 conjugation is not required per se to initiate Cul3 dimerization (the noncovalent interactions are sufficient), the populations of Cul3 dimer that are found in this study are composed of one neddylated and one unneddylated Cul3. We propose that under physiological conditions, the covalent attachment of Nedd8 ensures that Nedd8 will be present in the dimer. To directly measure how much Nedd8 conjugation strengthens the dimerization of the Cul3 complex, relative binding strength between Nedd8 and Cul3 was measured using the CPRG assay (Table 1). These data revealed that the binding interaction between Nedd8 and Cul3 is at least twice as strong when the lysine712 is available for neddylation rather than using only hydrophobic interactions with the Cul3K712R mutant.

There is also additional in vivo evidence that the covalent bond between cullins and Nedd8 is important but not essential for cullin function. A mutation in Cdc53 in *S. cerevisiae* revealed that the function of Cdc53 is reduced, but not eliminated, when the neddylation site (Rub1 addition) was mutated (Lammer *et al.*, 1998). This finding supports the dimer model because Cdc53 without its neddylation site could potentially maintain its dimerization via hydrophobic interactions with a Rub1 molecule and therefore remain functional. Thus, we propose that Nedd8 conjugation increases the stability of the Cul3 complex and makes the formation of the Cul3 dimer more efficient in vivo.

The Cul3 Heterodimer Is an Active Ubiquitin Ligase Complex

The current model of an active cullin-based ubiquitin ligase complex is derived from the crystal structure of the Cul1 complex, where the cullin is active as a monomer bound to a substrate adaptor protein at its N terminus and to a ubiquitin-conjugating enzyme at its C terminus (Zheng *et al.*, 2002). The crystal structure of Cul1 shows that the binding site for the substrate adaptor protein is on the opposite side of the binding site for the ubiquitin-conjugating enzyme, hindering the ability of the ubiquitin-conjugating enzyme to

reach its substrate. Based on our data, we propose an alternative model for an active Cul3 ubiquitin ligase complex in which the formation of a Cul3 dimer positions the BTB binding domain of one Cul3 molecule near the binding site of the ubiquitin-conjugating enzyme of its Cul3 partner. We predicted the head-to-head orientation for the two Cul3 proteins based on the orientation of the three helical bundle-containing dimer mediated by a ubiquitin molecule (Figure 7A). However, the findings from this study also support a head-to-tail orientation between the two Cul3 proteins (Figure 7B). The arrangement of the complex in either orientation would significantly reduce the distance between the substrates and the ubiquitin-conjugating enzyme, suggesting that Cul3 dimerization provides an alternative mechanism for substrate ubiquitination that could be adopted by the BCR ubiquitin ligase in response to specific sets of protein substrates.

The CSN Paradox Supports the Model That Dimerization Leads to the Activation of the Cul3 Complex

Studies involving a cullin deneddylation complex, CSN, revealed contradictory results known as the CSN paradox, which is briefly described as follows: It is known that ubiquitination activity of cullins is inhibited in vitro upon addition of the CSN complex (Zhou *et al.*, 2003). Thus, when a CSN subunit is knocked down by small interfering RNA, causing the inactivation of the CSN complex, cullin substrates should be degraded more rapidly due to increased cullin activity. However, the opposite result was observed: cullin substrates accumulated in vivo, indicating that cullin function is inhibited when CSN activity is reduced (Schwechheimer and Deng, 2000; Lykke-Andersen *et al.*, 2003; Pintard *et al.*, 2003a). Based on these data, it is thought that cullins require a cycle of both neddylation and deneddylation for proper function (Pintard *et al.*, 2003a; Parry and Estelle, 2004; Petroski and Deshaies, 2005). Although this model provides an explanation regarding the affect of Nedd8 conjugation and removal on the cullin-based ubiquitin ligase activity, it does not provide a precise role for Nedd8 in this complex. We propose an alternate model in which Nedd8 facilitates the formation of a Cul3 heterodimer, which is an active form of the Cul3-based ubiquitin ligase. Our model for the heterodimeric Cul3 complex offers a role for Nedd8 in cullin complex activation and accommodates the observations outlined by the CSN paradox. Because the Cul3 heterodimer is composed of one neddylated Cul3 and one unneddylated Cul3, enhancing Cul3 deneddylation by adding CSN complexes in vitro would inhibit dimer formation, causing the Cul3 complex to be inactive. When a subunit in the CSN complex is knocked down in vivo, the level of neddylated Cul3 increases, depleting the pool of unneddylated Cul3

required for dimer formation and inhibiting its ubiquitin ligase function.

The ability of Cul3 to function as a dimer may also have other biological significance. For example, the ability to convert a pool of inactive Cul3 monomer to a functional ubiquitin ligase complex by dimerization would shorten the activation time for the ubiquitin ligase and also allow cells to maintain a population of the Cul3 protein without constant production and degradation of Cul3 to control its ubiquitin ligase activity. If the Cul3 dimer is not the only active form of the BCR ubiquitin ligase, Cul3 dimerization could be a substrate-induced interaction to accommodate ubiquitination of target proteins other than those recognized by the Cul3 monomer. Furthermore, a functional Cul3 dimer may also provide an alternative mechanism for ubiquitin chain lengthening by alternating the substrate ubiquitination between the two Cul3 partners.

Dimerization Is Common among the Active Cullin-based Ubiquitin Ligase Complex

The ability of the Cul3 heterodimer to bind a BTB domain-containing protein and a substrate, cyclin E, suggests that the Cul3 heterodimer may be a functional ubiquitin ligase complex. Because all cullins share a conserved C-terminal domain that includes the WH-B domain, protein-protein interaction between Cul1 and Cul3 was tested in reciprocal immunoprecipitation assays, revealing that Cul3 was able to pull down Cul1 (Supplemental Figure 4). Because the C-terminal sequences of cullins are highly conserved and all cullins require neddylation to activate their ubiquitin ligase function, it is possible that other cullins also function as heterodimeric complexes. For example, Cul1 was pulled down by its substrate, I κ B, in a doublet that resembles a Cul3 heterodimer (Kawakami *et al.*, 2001). In addition, the Cul2 substrate HIF1 α immunoprecipitated Cul2 in doublets similar to the Cul3 dimer pulled down by cyclin E (Kawakami *et al.*, 2001; Ohh *et al.*, 2002), suggesting that other cullins are also active as dimers *in vivo*. Based on these observations, we suggest that dimerization is a common theme for cullin activation.

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REFERENCES

Angers, S., Thorpe, C. J., Biechele, T. L., Goldenberg, S. J., Zheng, N., MacCoss, M. J., and Moon, R. T. (2006). The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-beta-catenin pathway by targeting Dishevelled for degradation. *Nat. Cell Biol.* 8, 348–357.

Ciechanover, A., Heller, H., Elias, S., Haas, A. L., and Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proc. Natl. Acad. Sci. USA.* 77, 1365–1368.

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

Cope, G. A., Suh, G. S., Aravind, L., Schwarz, S. E., Zipursky, S. L., Koonin, E. V., and Deshaies, R. J. (2002). Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* 298, 608–611.

Geyer, R., Wee, S., Anderson, S., Yates, J., and Wolf, D. A. (2003). BTB/POZ domain proteins are putative substrate adaptors for Cullin 3 ubiquitin ligases. *Mol. Cell* 12, 783–790.

Glickman, M. H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* 82, 373–428.

Handeli, S., and Weintraub, H. (1992). The ts41 mutation in Chinese hamster cells leads to successive S phases in the absence of intervening G2, M, and G1. *Cell* 71, 599–611.

Hernandez-Munoz, I., Lund, A. H., van der Stoep, P., Boutsma, E., Muijers, I., Verhoeven, E., Nusinow, D. A., Panning, B., Marahrens, Y., and van Lohuizen, M. (2005). Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase. *Proc. Natl. Acad. Sci. USA* 102, 7635–7640.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479.

Hori, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., and Tanaka, K. (1999). Covalent modification of all members of human cullin family proteins by NEDD8. *Oncogene* 18, 6829–6834.

Kamitani, T., Nguyen, H. P., Kito, K., Fukuda-Kamitani, T., and Yeh, E. T. (1998). Covalent modification of PML by the sentrin family of ubiquitin-like proteins. *J. Biol. Chem.* 273, 3117–3120.

Kang, R. S., Daniels, C. M., Francis, S. A., Shih, S. C., Salerno, W. J., Hicke, L., and Radhakrishnan, I. (2003). Solution structure of a CUE-ubiquitin complex reveals a conserved mode of ubiquitin binding. *Cell* 113, 621–630.

Kawakami, T. *et al.* (2001). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J.* 20, 4003–4012.

Kurz, T., Pintard, L., Willis, J. H., Hamill, D. R., Gonczy, P., Peter, M., and Bowerman, B. (2002). Cytoskeletal regulation by the Nedd8 ubiquitin-like protein modification pathway. *Science* 295, 1294–1298.

Kwon, J. E. *et al.* (2006). BTB domain-containing speckle-type POZ protein (SPOP) serves as an adaptor of Daxx for ubiquitination by Cul3-based ubiquitin ligase. *J. Biol. Chem.* 281, 12664–12672.

Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., Callis, J., Goebel, M., and Estelle, M. (1998). Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCFCdc4 complex. *Genes Dev.* 12, 914–926.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D. A., Wei, N., and Deshaies, R. J. (2001). Promotion of NEDD8-CUL1 conjugate cleavage by COP9 signalosome. *Science* 292, 1382–1385.

Lykke-Andersen, K., Schaefer, L., Menon, S., Deng, X. W., Miller, J. B., and Wei, N. (2003). Disruption of the COP9 signalosome Csn2 subunit in mice causes deficient cell proliferation, accumulation of p53 and cyclin E, and early embryonic death. *Mol. Cell Biol.* 23, 6790–6797.

Mueller, T. D., and Feigon, J. (2002). Solution structures of UBA domains reveal a conserved hydrophobic surface for protein-protein interactions. *J. Mol. Biol.* 319, 1243–1255.

Ohh, M., Kim, W. Y., Moslehi, J. J., Chen, Y., Chau, V., Read, M. A., and Kaelin, W. G., Jr. (2002). An intact NEDD8 pathway is required for Cullin-dependent ubiquitylation in mammalian cells. *EMBO Rep.* 3, 177–182.

Ohta, T., Michel, J. J., and Xiong, Y. (1999). Association with cullin partners protects ROC proteins from proteasome-dependent degradation. *Oncogene* 18, 6758–6766.

Osaka, F., Kawasaki, H., Aida, N., Saeki, M., Chiba, T., Kawashima, S., Tanaka, K., and Kato, S. (1998). A new NEDD8-ligating system for cullin-4A. *Genes Dev.* 12, 2263–2268.

Ou, C.-Y., Lin, Y.-F., Chen, Y.-J., and Chien, C.-T. (2002). Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in *Drosophila* eye development. *Genes Dev.* 16, 2401–2414.

Pan, Z. Q., Kentsis, A., Dias, D. C., Yamoah, K., and Wu, K. (2004). Nedd 8 on cullin: building an expressway to protein destruction. *Oncogene* 23, 1985–1997.

Parry, G., and Estelle, M. (2004). Regulation of cullin-based ubiquitin ligases by the Nedd8/RUB ubiquitin-like proteins. *Semin. Cell Dev. Biol.* 15, 221–229.

Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* 6, 9–20.

Pines, J., and Lindon, C. (2005). Proteolysis: anytime, any place, anywhere? *Nat. Cell Biol.* 7, 731–735.

Pintard, L., Kurz, T., Glaser, S., Willis, J. H., Peter, M., and Bowerman, B. (2003a). Neddylation and deneddylation of Cul-3 is required to target MEI-1/Katenin for degradation at the meiosis-to-mitosis transition in *C. elegans*. *Curr. Biol.* 13, 911–921.

Pintard, L., Willems, A., and Peter, M. (2004). Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family. *EMBO J.* 23, 1681–1687.

- Pintard, L. *et al.* (2003b). The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. *Nature* 425, 311–316.
- Prag, G., Misra, S., Jones, E. A., Ghirlando, R., Davies, B. A., Horazdovsky, B. F., and Hurley, J. H. (2003). Mechanism of ubiquitin recognition by the CUE domain of Vps9p. *Cell* 113, 609–620.
- Robinson, D. N., and Cooley, L. (1997). *Drosophila* kelch is an oligomeric ring canal actin organizer. *J. Cell Biol.* 138, 799–810.
- Schwechheimer, C., and Deng, X. W. (2000). The COP/DET/FUS proteins-regulators of eukaryotic growth and development. *Semin. Cell Dev. Biol.* 11, 495–503.
- Schwechheimer, C., and Deng, X. W. (2001). COP9 signalosome revisited: a novel mediator of protein degradation. *Trends Cell Biol.* 11, 420–426.
- Seol, J. H. *et al.* (1999). 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.
- Sheaff, R. J., Singer, J. D., Swanger, J., Smitherman, M., Roberts, J. M., and Clurman, B. E. (2000). Proteasomal turnover of p21^{Cip1} does not require p21^{Cip1} ubiquitination. *Mol. Cell* 4, 403–410.
- Shih, S. C., Prag, G., Francis, S. A., Sutanto, M. A., Hurley, J. H., and Hicke, L. (2003). A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. *EMBO J.* 22, 1273–1281.
- Singer, J. D., Gurian-West, M., Clurman, B., and Roberts, J. M. (1999). Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. *Genes Dev.* 13, 2375–2387.
- Stogios, P. J., Downs, G. S., Jauhal, J. J., Nandra, S. K., and Prive, G. G. (2005). Sequence and structural analysis of BTB domain proteins. *Genome Biol.* 6, R82.
- Tateishi, K., Omata, M., Tanaka, K., and Chiba, T. (2001). The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice. *J. Cell Biol.* 155, 571–579.
- Torii, K. U., McNellis, T. W., and Deng, X. W. (1998). Functional dissection of *Arabidopsis* COP1 reveals specific roles of its three structural modules in light control of seedling development. *EMBO J.* 17, 5577–5587.
- Wada, H., Yeh, E. T., and Kamitani, T. (1999). Identification of NEDD8-conjugation site in human cullin-2. *Biochem. Biophys. Res. Commun.* 257, 100–105.
- Wilkinson, C. R., Seeger, M., Hartmann-Petersen, R., Stone, M., Wallace, M., Semple, C., and Gordon, C. (2001). Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. *Nat. Cell Biol.* 3, 939–943.
- Wu, K., Chen, A., and 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.
- Wu, K., Chen, A., Tan, P., and Pan, Z. Q. (2002). The Nedd8-conjugated ROC1-CUL1 core ubiquitin ligase utilizes Nedd8 charged surface residues for efficient polyubiquitin chain assembly catalyzed by Cdc34. *J. Biol. Chem.* 277, 516–527.
- Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W. L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. *Plant Cell* 14, 1919–1935.
- Xu, L., Yue, Y., Reboul, J., Vaglio, P., Shin, T.-H., Vidal, M., Elledge, S. J., and Harper, J. W. (2003). BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. *Nature* 425, 316–321.
- Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., and Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol. Cell Biol.* 24, 10941–10953.
- Zhang, Q., Zhang, L., Wang, B., Ou, C. Y., Chien, C. T., and Jiang, J. (2006). A Hedgehog-induced BTB protein modulates Hedgehog signaling by degrading Ci/Gli transcription factor. *Dev. Cell* 10, 719–729.
- Zheng, N. *et al.* (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* 416, 703–709.
- Zhou, C., Seibert, V., Geyer, R., Rhee, E., Lyapina, S., Cope, G., Deshaies, R. J., and Wolf, D. A. (2001). The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. *BMC Biochem.* 2, 7.
- Zhou, C., Wee, S., Rhee, E., Naumann, M., Dubiel, W., and Wolf, D. A. (2003). Fission yeast COP9/signalosome suppresses cullin activity through recruitment of the deubiquitylating enzyme Ubp12p. *Mol. Cell* 11, 927–938.
- Zhu, S., Perez, R., Pan, M., and Lee, T. (2005). Requirement of Cul3 for axonal arborization and dendritic elaboration in *Drosophila* mushroom body neurons. *J. Neurosci.* 25, 4189–4197.