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TIP120A associates with unneddylated cullin 1 and regulates its neddylation

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Abstract The cullin-containing E3 ubiquitin ligases play an important role in regulating the abundance of key proteins involved in cellular processes such as cell cycle and cytokine signaling. We recently identified TIP120A as a cullin-interacting protein and found that TIP120A functions as a negative regulator of a ubiquitin ligase by interfering with the binding of Skp1 and an F box protein to CUL1. Here we show that TIP120A binds to the unneddylated CUL1 but not the neddylated one. The association of TIP120A with CUL1 requires both the N-terminal stalk and the C-terminal globular domain of CUL1. TIP120A efficiently inhibits neddylation of CUL1 but does not affect substrate-independent ubiquitination by CUL1/Rbx1, implying that it blocks the access of Nedd8 to the conjugation site but does not interfere with the interaction of the ubiquitin-conjugating enzyme with Rbx1. Our data suggest that the association/dissociation of TIP120A coupled to neddylation/deneddylation of CUL1 may play an important role in assembly and disassembly of Skp1-Cdc53/cullin-F box ubiquitin ligases.

Key words: Nedd8; Neddylation; Ubiquitin; CUL1; Skp1-Cdc53/cullin-F box; E3 ubiquitin ligase

1. Introduction

The ubiquitin (Ub)-dependent degradation of regulatory proteins plays important roles in the control of various physiological processes such as cell cycle and signal transduction [1]. This process begins with the attachment of a multiubiquitin chain to a target protein, which involves the activities of Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes. The cullin family proteins are essential components in a group of multisubunit E3 Ub ligases and associate with the RING finger protein Rbx1 (also known as ROC1 and Hrt1) to form the integral core [2]. The Skp1-Cdc53/cullin-F box (SCF) complexes are the best characterized ones of this class of E3 ligases [3]. They consist of CUL1, Rbx1, Skp1, and an F box protein. Rbx1 interacts with the C-terminal domain of CUL1 and recruits the cognate E2 [4–6]. Skp1 functions as an adapter that links an F box protein to the N-terminal domain of CUL1 [7]. Substrates of the SCF complexes are bound by F box proteins, which contain the Skp1-binding F box motif and a variable protein–protein interaction domain that directly interacts with substrates [7,8]. A few SCF complexes have been demonstrated to have E3 activities for specific substrates such as β-catenin, IκBα, and $p27^{Kip1}$ [9,10]. Other members of the cullin family seem to function as a component of Ub E3 ligases [3]. Thus CUL2 assembles multisubunit E3 ligases that bear a striking resemblance to SCF-type complexes and directs ubiquitination of substrates, including α subunits of the hypoxia-inducible transcription factors HIF1 and HIF2 [11]. In addition, CUL3, CUL4A and CUL5 are shown to be involved in the degradation of specific substrates [12–14].

Cullin family proteins have been shown to be modified by Nedd8, a Ub-like small protein modifier [15,16]. Nedd8 modification of CUL1 activates ubiquitination of IκBα and $p27^{Kip1}$ [17–19], and enhances E2 Ub recruitment to SCF [20]. The Nedd8 pathway is essential for cell viability in fission yeast [21] and for the embryonic development in *Caenorhabditis elegans* [22] and in mice [23]. In *Arabidopsis thaliana*, Nedd8 modification is required for SCF-mediated auxin response [24,25]. Recent studies on the COP9 signalosome complex (CSN) have shown that CSN contains a deneddylating activity and have suggested that the cycles of neddylation and deneddylation may be needed to sustain optimal SCF activity [26,27].

In an effort to search for proteins regulating cullin-containing E3 ligases, we have recently identified TIP120A as a cullin-interacting protein and have shown that TIP120A functions as a negative regulator of $SCF^{\beta-TrCP}$ Ub ligase by interfering with the binding of Skp1 to CUL1 [28]. Here, we report that TIP120A inhibits neddylation of CUL1 and that Nedd8 conjugation of CUL1 prevents the binding of TIP120A to CUL1.

2. Materials and methods

2.1. Plasmids

The cDNAs encoding human Nedd8 (accession number BG396818), Uba3 (BC022853) and Ubc12 (BC007657) were obtained from Incyte Genomics Inc. To construct plasmids for the expression of CUL1 deletion mutants with an N-terminal FLAG tag, CUL1 cDNA was amplified by polymerase chain reaction with appropriate primers and ligated into pcDNA3.1(+) vector (Invitrogen).

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Abbreviations: Ub, ubiquitin; GST, glutathione *S*-transferase; HA, hemagglutinin; SCF, Skp1-Cdc53/cullin-F box; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2.2. Immunoprecipitations and Western blotting

Transfection was carried out by the $CaPO_4$-DNA precipitation method using HEPES or *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid buffer. After 36 h, cells were lysed in buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride, and 1.0% NP-40. Cell lysates were adjusted to 0.1% NP-40 and incubated with anti-FLAG antibody resin (Sigma) for 4 h at 4°C. The immune complexes were recovered by low speed centrifugation, and the resin was washed extensively with the binding buffer with 0.1% NP-40 and then eluted with buffer containing 20 mM Tris–HCl (pH 8.0) and 2% sodium dodecyl sulfate (SDS). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane (Bio-Rad) and visualized by Western blotting with the enhanced chemiluminescence reagents (Amersham Pharmacia). For Western blotting, we purchased commercial antibodies against FLAG (Sigma), hemagglutinin (HA) (Babco), TIP120A (BD Biosciences), CUL1 (Lab Vision), Rbx1 (Lab Vision), Ub (Covance) and Nedd8 (Alexis).

2.3. In vitro neddylation assays

To generate six-histidine-tagged Nedd8 and Ubc12, the cDNAs were cloned into pET15b (Novagen) and the proteins expressed in *Escherichia coli* strain BL21(DE3). The His-tagged proteins were purified using Co^{2+}-agarose beads (Clontech) according to the manufacturer's instructions. Glutathione *S*-transferase (GST)-Nedd8 was expressed in *E. coli* after cloning the cDNA into the pGEX4T-1 vector (Amersham Pharmacia Biotech) and purified by glutathione beads (Amersham Pharmacia Biotech) as recommended by the manufacturer. The FLAG-Uba3/APP-BP complex was purified from HeLa-derived cells stably transfected with an expression vector pYR-FLAG-hUba3. Nuclear extracts and cytosolic S100 extracts of HeLa cells were prepared as described previously [29]. Nuclear extracts were dialyzed against buffer BC (20 mM Tris–HCl (pH 7.9), 15% glycerol, 1 mM EDTA, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 0.05% NP-40) containing 150 mM KCl (BC150) and rotated with anti-FLAG M2-agarose (Sigma) at 4°C for 6 h. After extensive washes with BC150, FLAG-Uba3/APP-BP was eluted with 0.3 mg of FLAG peptide per ml in BC150. Generation of recombinant baculoviruses encoding human CUL1 with a six-His tag and human Rbx1 was previously described [30]. Sf21 cells were co-infected with the recombinant baculoviruses encoding His-CUL1 and Rbx1, and cell lysates were prepared as described [30]. The His-CUL1/Rbx1 complex was purified using Co^{2+}-agarose beads (Clontech) according to the manufacturer's instructions. Recombinant FLAG-His-tagged full-length TIP120A and its deletion mutants were expressed in Sf21 cells and purified as previously described [31].

The Nedd8 conjugation reaction (30 μl) contained 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM NaF, 100 nM okadaic acid, 2 mM ATP, 0.6 mM DTT, 1.0 μg of His-Nedd8 or GST-Nedd8, 50 ng of FLAG-Uba3/APP-BP, 200 ng of Ubc12, and 200 ng of His-CUL1/Rbx1. Reactions were incubated at 25°C for 90 min, terminated by adding 30 μl of 2×Laemmli loading buffer, and resolved by SDS–PAGE followed by Western blotting using anti-CUL1 antibodies to visualize CUL1 derivatives.

2.4. Protein–protein interaction assays

Recombinant His-CUL1/Rbx1 (600 ng) was neddylated in vitro using the purified Nedd8 conjugation system as described above. TIP120A (1 μg) was bound to 20 μl of anti-FLAG M2-agarose (Sigma) at 4°C for 2 h. Neddylated and unneddylated CUL1 was incubated with the M2-agarose beads covered with TIP120A in 100 μl of BC300 at 4°C for 2 h. After extensive washing with the same buffer, bound proteins were resolved by SDS–PAGE and analyzed by immunoblotting.

2.5. In vitro ubiquitination assays

His-tagged yeast Uba1, human UbcH5A and Ub were expressed in *E. coli* strain BL21(DE3) and purified by Ni^{2+}-agarose beads (Qiagen). The Ub ligation was performed in a 30 μl reaction containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM NaF, 100 nM okadaic acid, 2 mM ATP, 0.6 mM DTT, 0.8 μg of Ub, 50 ng of Uba1, 200 ng of UbcH5A, and 500 ng of His-CUL1/Rbx1. Reactions were incubated at 37°C for 20 min, terminated by adding 30 μl of 2×Laemmli loading buffer, and resolved by SDS–PAGE followed by Western blotting using anti-Ub antibodies to visualize high molecular mass Ub polymers.

3. Results and discussion

3.1. Mapping of domains in CUL1 required for the interaction with TIP120A

Recent structural studies on SCF^{Skp2} revealed that CUL1 is an elongated protein consisting of an N-terminal long stalk and a C-terminal globular domain [32]. The N-terminal stalk is composed of three repeats of a novel five-helix structural motif and the first repeat binds the Skp1-F box^{Skp2} complex. The C-terminal globular domain contains several structural motifs and assembles a complex with Rbx1 [32]. To map the structural features of CUL1 required for the interaction with TIP120A, we generated a series of expression vectors that direct the expression of FLAG-tagged CUL1 deletion mutants (Fig. 1A). These expression plasmids and an HA-TIP120A expression vector were transiently cotransfected into HeLa cells and anti-FLAG immunoprecipitation carried out on the cell lysates. The binding of TIP120A to CUL1 was monitored by Western blotting of immunoprecipitates with anti-HA antibodies (Fig. 1B). Deletion of the N-terminal 86 amino acids (CUL1NΔ86), which includes some of the critical Skp1-F box^{Skp2} binding residues [32], did not greatly affect TIP120A binding (lane 1). However, further deletions of the first repeat or more rendered CUL1 incapable of binding to TIP120A (lanes 4–7), suggesting that the first repeat of the N-terminal domain was required for TIP120A binding. Since the first repeat was shown to contain all the residues directly interacting with Skp1-F box^{Skp2} [32], these data implied that the TIP120A binding region of CUL1 overlapped the Skp1-F box^{Skp2} binding domain. This conclusion is consistent with our previous finding that the binding of TIP120A and Skp1 to CUL1 is mutually exclusive. Deletion of the C-terminal 31 amino acids of CUL1 (CUL1CΔ31) impaired its binding to TIP120A (lane 2). However, the CUL1CΔ31 mutant bound Rbx1, although more weakly than the full-length protein, suggesting that the deletion of the C-terminal 31 amino acids did not greatly affect the folding of the C-terminal domain. These results suggested that the winged-helix B (WH-B) motif of 70 amino acids in the C-terminal domain was essential for the binding of TIP120A. Collectively, the deletion analyses of CUL1 indicated that both the N-terminal stalk and the C-terminal globular domain contributed to the association of TIP120A.

3.2. Nedd8 modification of CUL1 prevents TIP120A binding

CUL1 is neddylated at Lys-720 in the WH-B motif. Since the WH-B motif appeared to be involved in TIP120A binding, we tested the possibility that neddylation of CUL1 could affect its association with TIP120A. HeLa cells were transiently transfected with expression constructs of FLAG-TIP120A, HA-CUL1 and HA-Rbx1, and anti-FLAG immunoprecipitation was performed. Western blotting of immunoprecipitates with anti-HA or anti-Nedd8 antibodies indicated that co-immunoprecipitated CUL1 was not modified with Nedd8 (Fig. 2A, lane 1). In contrast, both neddylated and unneddylated HA-CUL1 was co-purified in the immunoprecipitates of FLAG-Rbx1 following transient transfection with FLAG-Rbx1 and HA-CUL1 expression plasmids (lane 2). Although over-expression of TIP120A decreased the relative amount of

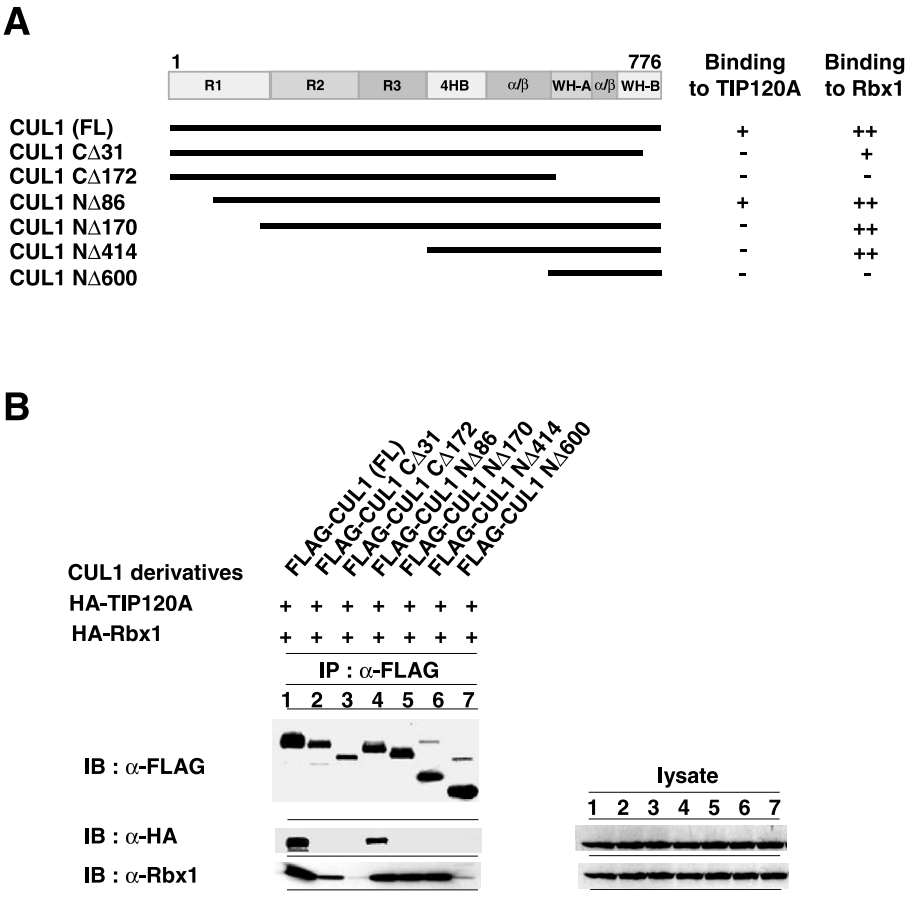

Fig. 1. Mapping of structural features in CUL1 required for the interaction with TIP120A. A: Schematic representation of full-length CUL1 and its deletion mutants. B: Immunoprecipitations of CUL1 derivatives. HeLa cells were transiently transfected with expression constructs for FLAG-tagged CUL1 derivatives. Cell lysates were immunoprecipitated with anti-FLAG antibody-coupled beads. The immunoprecipitates were subjected to SDS–PAGE and analyzed by Western blotting using anti-FLAG antibody to detect FLAG-tagged proteins, and anti-HA and anti-Rbx1 antibodies to detect co-immunoprecipitated proteins.

neddylated CUL1, it was present in the cell lysate (see Fig. 3A). In similar experiments, neddylated CUL1 was co-precipitated with β-TrCP1 or Skp1, whereas TIP120A immunoprecipitates always contained only unneddylated CUL1 (lanes 3 and 4, data not shown), suggesting that neddylation of CUL1 blocked the binding of TIP120A.

To confirm the inability of TIP120A to bind to neddylated CUL1, recombinant CUL1/Rbx1 complex was neddylated in vitro by a purified Nedd8 conjugation system consisting of APP-BP1/Uba3, Ubc12 and Nedd8, and incubated with recombinant FLAG-TIP120A bound to anti-FLAG antibody resin. The CUL1 bound to FLAG-TIP120A was removed by brief centrifugation and analyzed by immunoblotting with anti-CUL1 antibodies. As shown in Fig. 2B, most unneddylated CUL1 bound to beads, which was dependent on the presence of TIP120A in the reaction. In contrast, most neddylated CUL1 did not bind to beads but was found in the supernatant, confirming that neddylation of CUL1 impaired its binding to TIP120A.

3.3. TIP120A inhibits neddylation of CUL1

The inhibition of TIP120A binding by neddylation might result from the steric hindrance caused by Nedd8 conjugated to the WH-B motif on the protein binding surface. If so, we reasoned that prior binding of TIP120A might inhibit neddylation of CUL1. To test this possibility, HeLa cells were transfected with expression constructs of FLAG-CUL1 and Rbx1 with or without an HA-TIP120A expression plasmid, and anti-FLAG immunoprecipitation was carried out on the cell lysates. Immunoblotting of precipitates with anti-FLAG antibodies indicated that over-expression of TIP120A reduced the neddylated form of CUL1 by 60% as shown by immunoblotting with anti-Nedd8 antibodies (Fig. 3A, lanes 6 and 7). The decrease of neddylated CUL1 by TIP120A was specific since over-expression of DDB1, which does not bind to CUL1, did not affect neddylation of CUL1 (lanes 4 and 8). These data suggest that TIP120A negatively regulated CUL1 neddylation.

To directly show whether TIP120A inhibits neddylation of CUL1, recombinant TIP120A, its deletion mutants and DDB1 were expressed in Sf21 cells and purified by an immunoaffinity purification method (Fig. 3B). Purified proteins were added to in vitro neddylation reactions reconstituted with recombinant components. Six-His-tagged Nedd8 was efficiently conjugated to recombinant CUL1 (Fig. 3C, lanes 2 and 6). Addition of recombinant TIP120A inhibited conjugation of Nedd8 to CUL1 in a dose-dependent manner (lanes 2–5). The inhibition by TIP1210A was very efficient and almost complete at a 1:1 molar ratio of TIP120A to CUL1 (lane 4). Furthermore, DDB1 and deletion mutants of TIP120A incapable of binding to CUL1 did not affect CUL1 neddylation (lanes 8–10). These data demonstrate

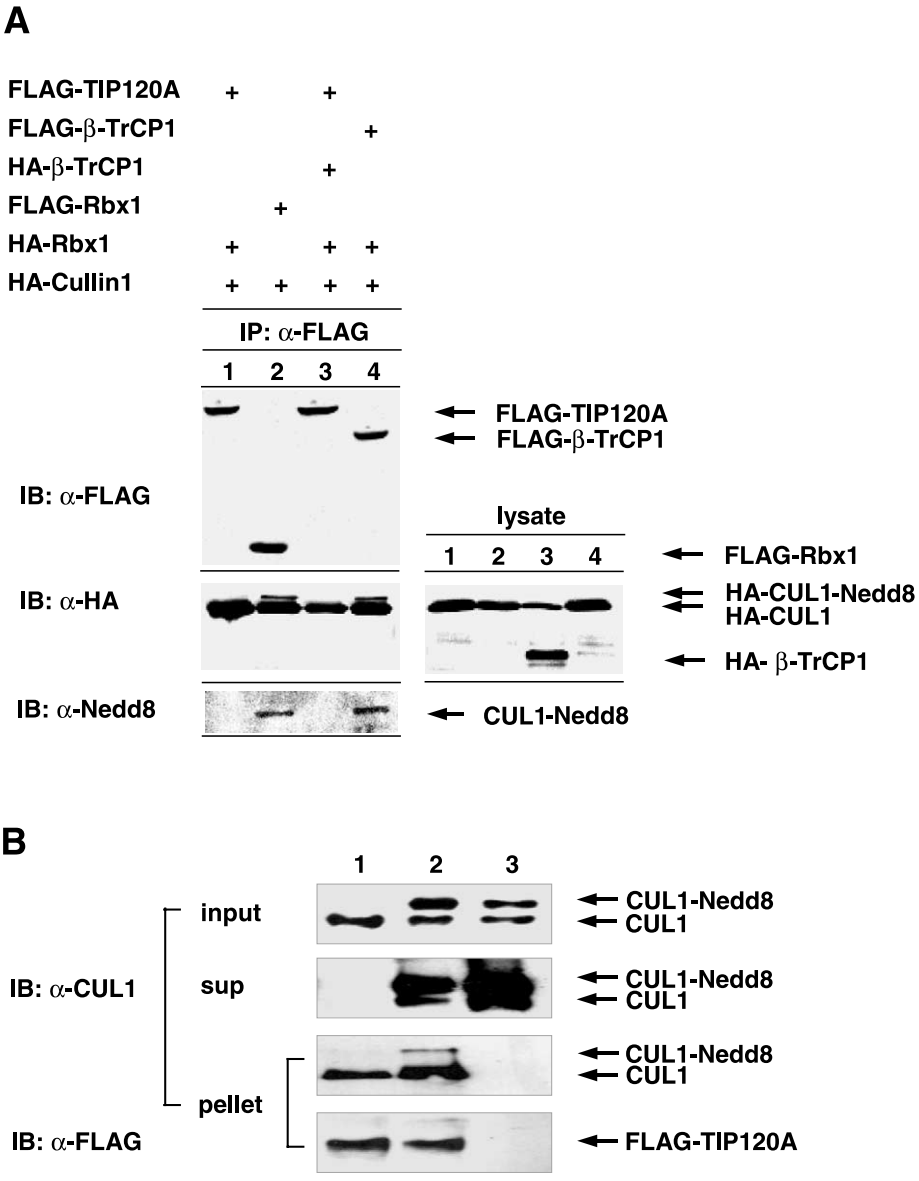

Fig. 2. Inability of TIP120A to bind neddylated CUL1. A: Co-precipitation of TIP120A with unneddylated CUL1. HeLa cells were transiently transfected with plasmid vectors expressing epitope-tagged TIP120A, CUL1, Rbx1 and β-TrCP1 proteins in combinations as indicated. Immunoprecipitations with α-FLAG antibodies and immunoblotting experiments were performed as described in Fig. 1. B: In vitro protein–protein interaction assay. Recombinant His-CUL1/Rbx1 was either untreated (lane 1) or neddylated in vitro (lanes 2 and 3) as described in Section 2, and incubated with anti-FLAG M2-agarose beads either untreated (lane 3) or pre-bound by TIP120A (lanes 1 and 2). Following removal of the beads by brief centrifugation, an aliquot of each fraction was analyzed by immunoblotting using indicated antibodies.

that inhibition of neddylation by TIP120A was specific and required its binding to CUL1.

3.4. *TIP120A does not affect substrate-independent polyubiquitination by CUL1/Rbx1*

Conceivably, the binding of TIP120A might block not only the access of Nedd8 to the conjugation site Lys-720 on CUL1 but also that of Ubc12-Nedd8 to Rbx1, which was thought to activate E2. To examine whether TIP120A might inhibit the interaction of E2s to Rbx1 by binding to CUL1/Rbx1, we devised an in vitro substrate-independent polyubiquitination assay using recombinant His-CUL1/Rbx1 as the E3 and tested the effect of TIP120A on ubiquitination (Fig. 4). His-CUL1/Rbx1 supported efficient polyubiquitination, which was dependent on Ubc5A (lanes 1 and 2). Polyubiquitination was not inhibited by full-length TIP120A or by a deletion mutant incapable of CUL1 binding (lanes 3–6). Since TIP120A did not have any effect on the polyubiquitination at a TIP120A/CUL1 molar ratio of 3 (lane 5), at which it completely inhibited CUL1 neddylation (Fig. 3C, lanes 5 and 7), we concluded that most CUL1 formed a complex with TIP120A under the conditions used and that the binding of TIP120A to His-CUL1/Rbx1 did not prevent the interaction of Ubc5A with Rbx1. It was previously reported that coexpression of CUL1 and Rbx1 in Sf21 cells resulted in neddylation of a fraction of CUL1 [33]. Although we could not detect neddylated CUL1 in the CUL1/Rbx1 preparation (see Fig. 3C, lane 1), it was formally possible that a tiny amount of neddylated CUL1/Rbx1 which did not bind TIP120A directed the polyubiquitination reaction shown in Fig. 4. To exclude this pos-

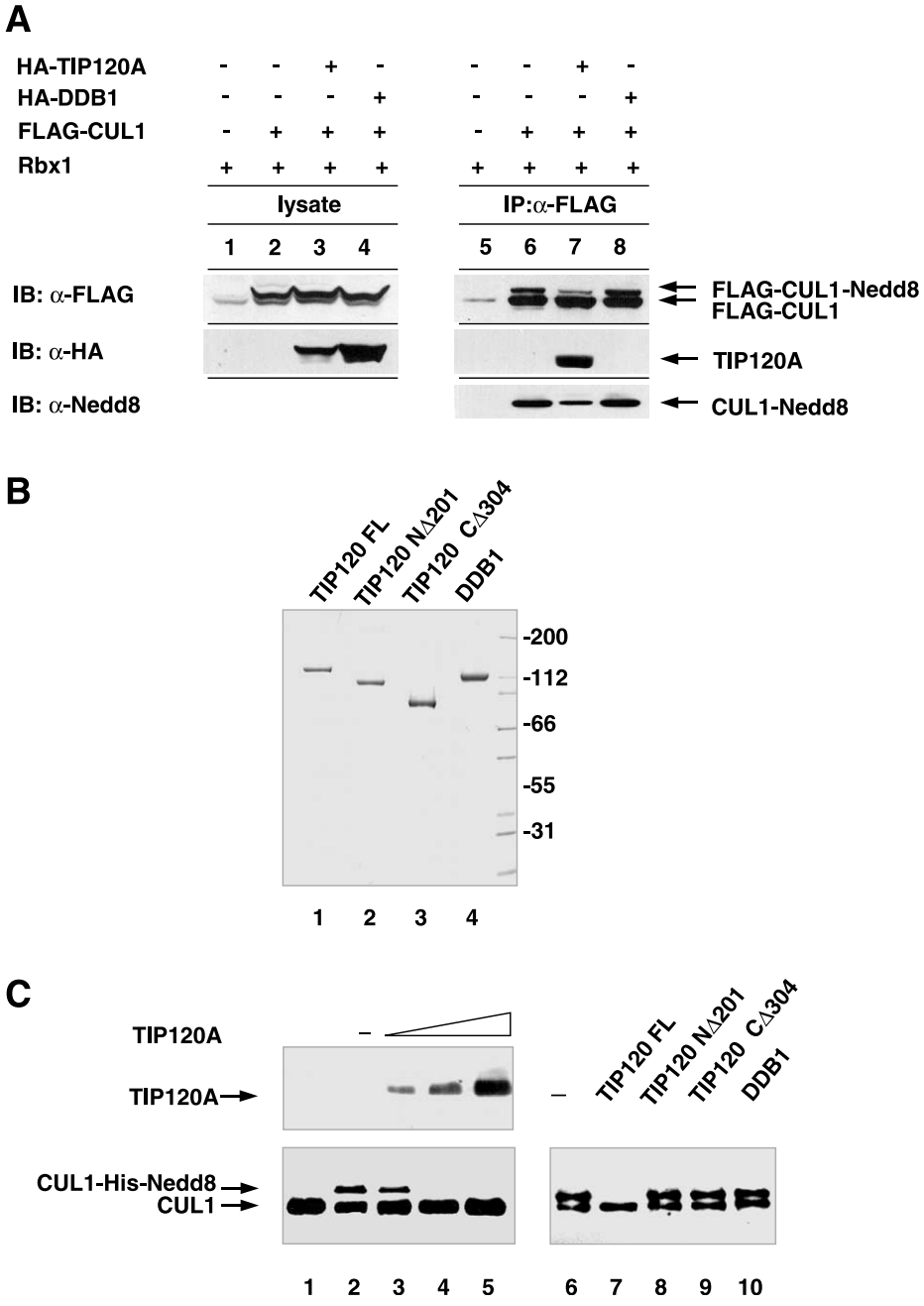

Fig. 3. Inhibition of CUL1 neddylation by TIP120A. A: Immunoprecipitation of CUL1 following over-expression of TIP120A. HeLa cells were transiently transfected with plasmid vectors expressing HA-TIP120A, HA-DDB1, FLAG-CUL1 and Rbx1 in combinations as indicated. Immunoprecipitations and immunoblotting with indicated antibodies were performed as described in Fig. 1. B: SDS–PAGE analysis of purified TIP120A and its deletion mutant proteins. Recombinant proteins were expressed in Sf21 cells and purified as described in Section 2. Two μl each of purified proteins was separated by 10% SDS–PAGE and visualized by Coomassie blue staining. Protein size markers (in kDa) are indicated on the right. C: In vitro neddylation assays. Recombinant His-CUL1/Rbx1 was either untreated (lane 1) or neddylated in vitro (lanes 2–10) as described in Section 2 using His-Nedd8. The molar ratio of TIP120A to CUL1 was approximately 0.3 (lanes 3 and 7), 1 (lanes 4 and 8), or 3 (lanes 5, 7–10). NΔ201 and CΔ304 represent TIP120A mutants with the truncation of N-terminal 201 and C-terminal 304 amino acids, respectively. Reactions were terminated by adding Laemmli loading buffer and resolved by SDS–PAGE followed by immunoblotting with anti-CUL1 antibodies.

sibility, HeLa cells were transfected with expression constructs of FLAG-TIP120A, HA-CUL1 and HA-Rbx1. The FLAG-TIP120A-containing complex was immunopurified using anti-FLAG antibody resin and tested for polyubiquitination. Although the FLAG-TIP120A immunoprecipitates contained only the unneddylated form of CUL1 (see Fig. 2A), it efficiently directed polyubiquitination (lane 8). These results strongly suggest that TIP120A did not block the interaction of the E2 with Rbx1.

We have recently identified TIP120A as a cullin-interacting protein and found that it functions as a negative regulator of an SCF Ub ligase by interfering with the binding of Skp1 and an F box protein [28]. Here we have extended our understanding of TIP120A function by showing the regulation of

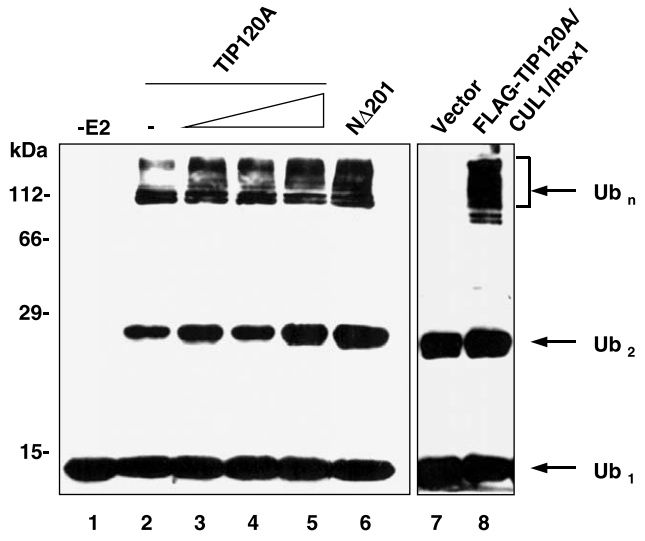

Fig. 4. Effect of TIP120A on substrate-independent polyubiquitination. In vitro ubiquitination reactions were performed as described in Section 2 using recombinant His-CUL1/Rbx1 (lanes 1–6) or anti-FLAG immunoprecipitates (lanes 7,8) as an E3 Ub ligase. The molar ratio of TIP120A to CUL1 was approximately 0.3 (lane 3), 1 (lane 4), or 3 (lanes 5,6). Reactions were terminated by adding Laemmli loading buffer and resolved by SDS–PAGE followed by immunoblotting with anti-Ub antibodies.

TIP120A binding by neddylation of CUL1. Based on the data presented here, we believe that TIP120A touches at least two domains in CUL1, namely N-terminal R1 and C-terminal WH-B motifs. In addition, we propose that binding of TIP120A to CUL1 blocks the access of Nedd8 to the conjugation site and that neddylation of CUL1 inhibits TIP120A binding by introducing a bulky domain on the protein interaction surface.

Presumably, most of the unneddylated CUL1 is present in the cell as a trimeric complex with TIP120A and Rbx1, which has no E3 ligase activity towards specific substrates. Although it is unclear how TIP120A is dissociated from unmodified CUL1, we expect that once CUL1 is neddylated, modified CUL1 no longer binds TIP120A and forms functional SCF complexes with Skp1 and a variety of F box proteins. Following ubiquitination and proteolysis of the specific substrate, CUL1 in the SCF complex is presumed to be deneddylated by CSN, which would lead to disassembly of the SCF complex by TIP120A and eventual recycling of CUL1. This scenario is consistent with the notion that Nedd8 needs to be cyclically attached to and cleaved from CUL1 for optimal SCF function [26], and introduces a new idea that the association/dissociation of TIP120A coupled to neddylation/deneddylation of CUL1 plays an important role in assembly and disassembly of SCF Ub ligases.

While we were preparing the manuscript, two groups reported the regulation of SCF Ub ligases by CAND1/TIP120A [34,35]. Our data agree with their findings in that CAND1/TIP120A selectively associates with unneddylated CUL1 and further show that CAND1/TIP120A inhibits neddylation of CUL1 in a fully defined in vitro system while it does not affect substrate-independent ubiquitination by CUL1/Rbx1. Whereas Xiong's group showed that CAND1/TIP120A-associated CUL1 was conjugated by NEDD8 in a partially purified system [34], we were not able to detect CUL1 neddylation in the presence of excess CAND1/TIP120A in our highly purified system. These seemingly contradictory results could be resolved by presumed additional factor(s) which may be required for dissociation of CAND1/TIP120A from CUL1. Interestingly, in this regard ATP was reported to regulate dissociation of CAND1/TIP120A independently of CUL1 neddylation in HeLa extract [35]. Therefore, the factor necessary for dissociation of CAND1/TIP120A from CUL1 may require ATP for its function.

CAND1/TIP120A binds most cullins that are known to be modified by Nedd8. Thus, CAND1/TIP120A may also modulate other cullin-containing Ub ligases. Further studies are needed to clarify the role of CAND1/TIP120A in the regulation of cullin-containing Ub ligases.

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