The Ubiquitin Conjugating Enzyme, UbcM2, Engages in Novel Interactions with Components of Cullin-3 Based E3 Ligases[†]

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ABSTRACT: The class III ubiquitin conjugating enzymes (E2s) are distinguished from other E2s by the presence of unique N-terminal domains, and the utilization of importin-11 for transport into the nucleus in an activation dependent fashion. To begin determining the physiological roles of these enzymes, we carried out a yeast two-hybrid screen with the class III E2, UbcM2. This screen retrieved RCBTB1, a putative substrate adaptor for a cullin3 (CUL3) E3 ligase. We initially established through biochemical studies that RCBTB1 has the properties of a CUL3 substrate adaptor. Further analysis of the UbcM2-RCBTB1 complex led to the discovery and characterization of the following novel interactions: (i) UbcM2 binds an N-terminal domain of CUL3 requiring the first 57 amino acids, the same domain that binds to RCBTB1 and other substrate adaptors; (ii) UbcM2 does not bind mutants of CUL3 that are deficient in substrate adaptor recruitment; (iii) UbcM2 interacts with CUL3 independent of a bridging RING-finger protein; and (iv) can engage the neddylated (i.e., activated) form of CUL3. We also present evidence that UbcM2 can bind to the N-terminal halves of multiple cullins, implying that this E2 is a general cofactor for this class of ligases. Together, these studies represent the first evidence that UbcM2, in concert with substrate adaptors, engages activated CUL3 ligases, thus suggesting that class III E2s are novel regulators of cullin ligases.

Ubiquitin is a small, highly conserved, eukaryotic protein that is post-translationally attached to substrates. Proteins tagged with ubiquitin can be targeted to the 26S proteasome for degradation or marked for nonproteolytic outcomes such as transcriptional activation, endocytic sorting, and DNA repair (reviewed in refs *1*−*3*). In vivo, most ubiquitin conjugation is mediated by a hierarchical, three-enzyme cascade consisting of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Ubiquitin is first activated in an ATP-dependent manner by E1. The activated ubiquitin is then transferred to the active site cysteine of an E2 in a transesterification reaction. E2s typically partner with an E3 to transfer the activated ubiquitin to a substrate but can also monoubiquitylate ubiquitin-binding domains independent of a partnering E3 (*4*). E3s can be single proteins or multisubunit complexes, and they confer substrate selection and specificity to the ubiquitin system. They can be classified into two superfamilies, really interesting new gene (RING)-finger E3s and homologous to E6-AP carboxy terminus (HECT) E3s.

Following the discovery that proteins containing RING-finger domains can function as E3[1] ligases (*5*), it was immediately appreciated that eukaryotic cells likely contain hundreds of distinct E3s. A family of E3s with strong conservation throughout eukaryotic evolution and broad biological relevance are the cullin-RING E3 ligases (CRLs). These are the largest class of E3 ligases, and they regulate a wide range of cellular and developmental processes (reviewed in ref *6*). Crystallographic (e.g., refs *7*−*10*) and biochemical analyses (e.g., refs *11*−*15*) have revealed a general model for the architecture and function of CRLs. These multisubunit E3s have a catalytic core comprised of a cullin (CUL) bound through its C-terminal domain to a RING-finger protein (Roc1/Rbx/Hrt1). The RING-finger recruits a ubiquitin-charged E2 (*13, 14*), promotes cullin accumulation in the nucleus (*16*), and enhances modification of cullins with the ubiquitin-like molecule, neural-precursor-cell-expressed and developmentally downregulated 8,

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[1] Abbreviations: E2, ubiquitin conjugating enzyme; CUL, cullin; RING, really interesting new gene; E1, ubiquitin-activating enzyme; E3, ubiquitin ligase; HECT, homologous to E6-AP carboxy terminus; CRL, cullin-RING E3 ligases; Nedd8, neural-precursor-cell-expressed and developmentally downregulated 8; Skp1, S-phase kinase-associated protein 1; BTB, broad complex, Tramtrack, bric-a-brac; TNT, coupled in vitro transcription and translation; GSH, glutathione; GST, glutathione-*S*-transferase; HEK293T, human embryonic kidney 293T cells; HA, hemagglutinin; UBC, E2 catalytic core domain; GFP, green fluorescent protein; IPTG, isopropyl-*β*-D-thiogalactopyranoside; 3-AT, 3-aminotriazole; HRP, horseradish-peroxidase; ATS1p, alpha-tubulin suppressor protein; YFP, yellow fluorescent protein; MW, molecular weight; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol tetraacetic acid; Ub, ubiquitin.

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Nedd8 (*11, 16*−*18*). The N-terminal domain of the CUL binds to one or more adaptor proteins which in turn recruit substrates for ubiquitylation (*19*). Regulation of CRL activity is influenced by multiple factors including dimerization (*20*), ubiquitylation and deubiquitylation of CRL components (e.g., refs *7*, *21*−*23*) and the dynamic attachment and removal of Nedd8 to a conserved lysine in the carboxy-terminal domain of the CULs (e.g., refs *24*−*26*). Nedd8 is conjugated to CULs by a dedicated set of enzymes and is removed by a multisubunit complex called the COP9 signalosome (reviewed in refs *27*−*29*). Thus, CRL regulation is a multifaceted process governed by an array of proteins and enzymatic complexes.

Humans have at least eight CULs (CUL1, 2, 3, 4A, 4B, 5, 7, and PARC), and each typically interacts with a unique type of substrate adaptor (*30*). For example, CUL1 interacts with a substrate adaptor termed Skp1. Skp1 in turn recruits a binding partner containing an F-box domain. The F-box protein binds to the substrate and presents it to the CUL-associated E2 for ubiquitin modification. The use of substrate adaptors composed of two proteins is also employed by CUL2, CUL4, CUL5, and CUL7 (all reviewed in ref *27*). In contrast, CUL3 complexes recruit substrates through single polypeptide adaptors that have BTB domains (*19, 21, 31*). The BTB domain makes direct contact with CUL3, and the other end of the adaptor often contains a protein−protein interaction motif such as a Kelch repeat or a MATH domain that binds to the substrate. The capacity of CRLs to regulate large numbers of proteins is reflected by the estimate that the human genome encodes hundreds of BTB domain-containing proteins (*19*), suggesting that CUL3 CRLs alone mediate the ubiquitylation of at least this many substrates.

Open questions remain regarding the molecular details of CRL regulation. For example, what roles do E2s play in modulating the various CRL components? How do CULs with large numbers of substrate adaptors select among these adaptors in temporally and spatially appropriate ways? In this report, we have identified a new mechanism by which particular E2s can interact with cullins. Specifically, we have demonstrated that the highly conserved metazoan E2, UbcM2, is recruited to the N-terminal end of CUL3 through the same domain that recruits BTB substrate adaptors. Unlike the prototypical association of E2s with CUL3, UbcM2 recruitment does not require a bridging RING-finger protein but does require substrate adaptor recruitment. The consequences of this novel interaction are that UbcM2 can engage Nedd8-modified CUL3 complexes (i.e., activated) and can monoubiquitylate CUL3. We further demonstrate that UbcM2 associates with the N-terminal half of CUL1 and CUL4A, implicating the enzyme as a general cofactor of cullin-based E3 ligases. We also provide evidence that engagement of the N-terminal half of CUL3, and perhaps other cullins, is a property shared by all three class III E2s. Additionally, we show that RCBTB1, a protein genetically linked to hematological malignancies (*32*), has the properties of a CUL3 substrate adaptor. These studies represent the first evidence that the class III ubiquitin conjugating enzymes have previously unrecognized and unique roles in modulating CUL3-based E3 ligases.

MATERIALS AND METHODS

Binding Assays. Proteins were expressed by *in vitro* transcription/translation (TNT) using rabbit reticulocyte lysates (Promega) and expression plasmids harboring either SP6 or T7 promoters. TNT reactions included an amino acid mixture lacking methione but containing ^{35}S-translabel (mixture of methionine and cysteine). Reactions were incubated at 30 °C for 1.5 h immediately prior to being used in binding or ubiquitylation reactions. Binding reactions were typically done by combining the ^{35}S-labeled proteins with bacterially expressed and purified recombinant fusion proteins at 4 °C for 3 h. Complexes were captured on either glutathione (GSH)-sepharose beads, S-protein agarose beads, or Flag-agarose beads, washed with at least 20 column volumes of binding buffer (10 mM HEPES-KOH [pH 7.4], 55 mM potassium acetate, 1 mM magnesium acetate, 0.1 mM EGTA, 0.25% Tween-20, 150 mM NaCl), and resolved by SDS−PAGE. Gels were stained with Coomassie Brilliant Blue (CBB) to visualize the recombinant proteins, and subsequently treated with 1 M sodium salicylate in water for 30 min prior to being dried down onto Whatman paper and exposed to X-OMAT film at −80 °C (fluorography) or at room temperature (direct exposure, autoradiography) to detect the ^{35}S-labeled proteins. Where indicated, binding reactions consisted of two ^{35}S-labeled proteins expressed individually in TNT reactions and then combined with the indicated matrix specific for a tag present on one of the proteins. For Figure 1E, the binding of UbcM2 to each of the ^{35}S-labeled, RCBTB1 proteins was quantitated by simultaneously exposing gels from three independent experiments to a phosphorimager screen. To normalize values, ^{35}S-band intensity was divided by the number of methiones in each protein.

Recombinant Protein Expression. Recombinant fusion proteins were expressed in BL21(star) *Escherichia coli* grown in terrific broth supplemented with 2% ethanol and the appropriate antibiotic. Cultures were grown at 37 °C until the optical density at 600nm reached 0.8−1.0. Recombinant protein expression was then induced by supplementing cultures with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and growing the cultures for 14−18hrs at 23 °C. Bacteria were collected by centrifugation, resuspended in lysis buffer, lysed using an Emulsiflex-C5 homogenizer (Avestin, Inc., Ottawa, Canada), and rocked with the appropriate bead matrix. Purified proteins were recovered by elution and exchanged into Microinjection buffer (10 mM $NaPO_4$ [pH 7.2], 70 mM KCl) supplemented with 150 mM NaCl.

Cloning. RCBTB1 was cloned from a human testes cDNA library using sequence-specific PCR primers. RCBTB2 was cloned from HeLa cell cDNA using sequence-specific PCR primers. The sequences of both cDNAs were verified by DNA sequencing.

Yeast Two-Hybrid Assays. (C145S) UbcM2 fused to the DNA binding domain of the GAL4 protein was used as the bait for a yeast two-hybrid screen of a randomly primed, cDNA library created from a 10-day mouse embryo. Prey proteins were expressed as fusions with the transactivation domain of the herpesvirus VP16 protein. Positives were selected on Leu, Trp, His triple dropout plates containing 10 mM 3-aminotriazole. Prey plasmids were recovered and

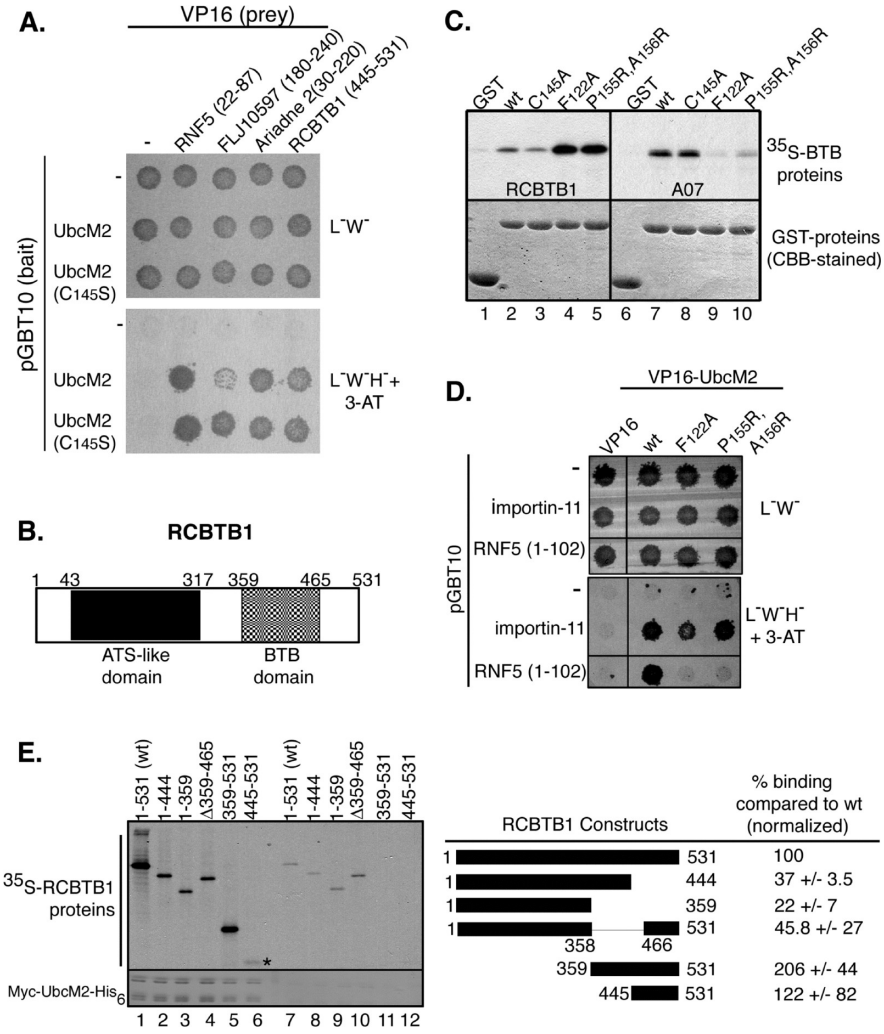

FIGURE 1: UbcM2 interacts with a C-terminal domain of RCBTB1 in a RING-finger protein-independent fashion. (A) Directed yeast two-hybrid results using wild type or catalytically inactive (C145S) UbcM2 as the bait and fragments from 4 putative E3 ligases as the prey. Top panel shows a photograph of a plate lacking leucine and tryptophan (L^-W^-) on which yeast were mated. Bottom panel is a photograph of a plate lacking leucine, tryptophan, and histidine but containing 10 mM 3-aminotriazole ($L^-W^-H^-$ + 3-AT). The $L^-W^-H^-$ + 3-AT plate was replica plated from the L^-W^- mating plate. (B) Schematic of RCBTB1. The ATS-like domain (black rectangle) spans residues 43 to 317 and has 39% homology with ATS1p, the alpha-tubulin suppressor protein from *S. cerevisiae*. Amino acids 359−465 comprise the BTB domain (patterned box). The numbers represent amino acid positions. (C) UbcM2 interacts with RCBTB1 independent of a bridging RING-finger protein. Parallel GST-fusion protein pulldowns of ^{35}S-labeled RCBTB1 or ^{35}S-labeled AO7 expressed by TNT. Fractions of bead-bound proteins (50% total) were resolved by SDS−PAGE. GST proteins were visualized by CBB-staining (bottom panels) and ^{35}S-labeled RCBTB1 and AO7 by fluorography (top panels, lanes 1−5 and 6−10, respectively). Point mutants of GST-UbcM2 are denoted above lanes 3, 4, 5, 8, 9, and 10. (D) Directed yeast two-hybrid assay to demonstrate that the F122A and P155R, A156R mutants of UbcM2 are deficient in binding to a RING-finger domain from RNF5. The RING-finger domain of RNF5 resides between residues 22 and 87. As a positive control, all UbcM2 proteins interacted with the transport receptor importin-11. Diploid yeast were grown on L^-W^- plates and replica plated onto $L^-W^-H^-$ + 3-AT plates to detect protein−protein interactions. (E) Recombinant Myc-UbcM2-His$_6$ pulldown of the indicated ^{35}S-labeled RCBTB1 proteins expressed in individual TNT reactions. Myc-UbcM2-His$_6$ and any associated ^{35}S-labeled proteins were precipitated with 9E10 monoclonal antibody and protein-A sepharose. Myc-UbcM2-His$_6$ was visualized following SDS−PAGE by CBB staining (bottom panel, lanes 1−6) and the ^{35}S-labeled RCBTB1 proteins were visualized by fluorography. Lanes 7−12 represent negative controls lacking the 9E10 monoclonal antibody. A schematic of each RCBTB1 construct is shown on the right with amino acid numbers. Binding was quantitated from 3 independent experiments, and band intensity was normalized to the number of methionines in each protein. % binding of each protein compared to wild type RCBTB1 is listed with standard deviations. Asterisk next to lane 6 marks a band representing residues 445−531 which contains only a single methionine.

retested in directed yeast two-hybrid assays following transformation into a *Saccharomyces cerevisiae* W303a (MATα) strain. For these assays, the UbcM2-encoding bait plasmid was transformed into a *S. cerevisiae* HF7c (MATa) strain. The prey plasmids from confirmed interactions were sent for DNA sequencing. Yeast two-hybrid screens done using RCBTB1 (wt or residues 1−359) were performed by the Indiana University Yeast Two-Hybrid facility (http://www.bio.indiana.edu/~michaelslab/yeast_two_hybrid_facility.html).

Western Blotting. Proteins were resolved by SDS−PAGE and electrotransferred to nitrocellulose, and blots were blocked in 5% nonfat milk/TBST (0.1% Tween-20 in Tris-buffered saline). The anti-Flag M2 monoclonal antibody (Sigma) was diluted 1:2500 in 5% nonfat milk/TBST, and the anti-HA 12CA5 monoclonal antibody (*33*) was diluted 1:1000. A goat antimouse horseradish-peroxidase (HRP)-conjugated secondary antibody from Jackson Laboratories was diluted 1:20,000. To detect the S-peptide, S-protein-HRP from Novagen was diluted 1:5000 in TBST. Enhanced

chemiluminescence (KPL) was carried out according to the manufacturer's instructions.

Transfections, Immunofluorescence and Fluorescence Microscopy. HEK293T cells were transfected by the calcium phosphate method (*34, 35*). Overexpressed cullins were triple hemagglutinin-tagged (HA^3), and RCBTB1 was either Myc- or Flag-tagged. 2−3 days post-transfection, cells were lysed in binding buffer [10 mM HEPES-KOH [pH 7.4], 55 mM potassium acetate, 1 mM magnesium acetate, 0.1 mM EGTA, 0.25% Tween-20, 150 mM NaCl] by incubation for 15 min on ice. Lysates were clarified by centrifugation and combined with either recombinant proteins or antibodies to recover complexes. For immunofluorescence studies (Supplemental Figure 2 in the Supporting Information), HeLa cells were plated on glass coverslips, transfected by the calcium phosphate method, and 2 days later processed for immunofluorescence and fluorescence microscopy. Cells were fixed in 3.7% formaldehyde in PBS, permeabilized in −20 °C methanol for 2 min at room temp, and blocked in 3% BSA/PBS. Immunostaining of Myc-RCBTB1 was done using an anti-Myc 9E10 monoclonal antibody (*33*) at a dilution of 1:500 followed by a goat antimouse $Alexa_{546}$ secondary antibody diluted 1:4000 in 3% BSA/PBS. YFP-RCBTB2 was examined by epifluorescence microscopy. Cells were examined with a Nikon TE2000 inverted, epifluorescence microscope equipped with a 60× water immersion objective (NA 1.20). Images were captured with a Hamamatsu Orca AG charge-cooled digital camera controlled by Openlab software (Improvision, Inc.) and pseudocolored and prepared for figures using Adobe Photoshop (version 8.0).

RESULTS

The human class III E2s (UBE2E1, UBE2E2, and UBE2E3) engage in complexes with damaged DNA binding protein 1 and de-etiolated 1, substrate adaptor components for the CUL4a E3 ligases (*36*). However, the functions of the enzymes in these complexes remain to be determined. It is also not known whether the class III E2s function with other cullins. To gain further insight into these issues, we carried out a yeast two-hybrid screen with UbcM2, the mouse counterpart of human UBE2E3. The two enzymes are 100% identical. Four interacting proteins were identified, and each interacted with both wild type and catalytically inactive (C145S) UbcM2 (Figure 1A). The enzyme interacted with the RING-finger domain-containing fragments of FLJ10597, RNF5, and Ariadne-2 (Figure 1A). The other prey, RCBTB1, is a BTB domain-containing protein genetically implicated in hematological malignancies (*32*). Three independent, overlapping clones of RCBTB1 were retrieved with the smallest common domain consisting of the carboxy-terminal 87 amino acids, residues 445−531 (Figure 1A). This region partially overlaps with the BTB domain (residues 359−465 (Figure 1B)).

We used binding studies to characterize the interaction between RCBTB1and UbcM2. The human form of RCBTB1 is 96% identical to its mouse counterpart and was used for all subsequent experiments. ^{35}S-RCBTB1 was expressed in a rabbit reticulocyte lysate by TNT and combined with different forms of GST-UbcM2. The C145A mutant is catalytically inactive (*33*), and the F122A and P155R/A156R mutants are deficient in E3 binding (Figures 1C and 1D and refs *37*, *38*). These experiments confirmed that wild type and catalytically inactive UbcM2 bound full-length RCBTB1 (Figure 1C, lanes 2 and 3). Unexpectedly, the F122A and P155R/A156R UbcM2 mutants interacted with RCBTB1 more robustly than wild type enzyme (Figure 1C, lanes 4 and 5). In contrast, neither mutant appreciably precipitated the RING-finger protein AO7 (Figure 1C, lanes 9 and 10), nor interacted with the RING-finger protein RNF5 (Figure 1D). Expression and proper folding of the mutants was demonstrated by their interaction with importin-11 (Figure 1D). Together, these data show that UbcM2 interacts with full-length RCBTB1 independent of the active site cysteine and that UbcM2 mutants which do not productively associate with RING-finger containing proteins have an increased affinity for RCBTB1. This prompted us to test the idea that RING-finger proteins and RCBTB1 bind mutually exclusively to UbcM2. We tested this by attempting to dissociate a bead-bound UbcM2 /^{35}S-RCBTB1 complex with a large molar excess of recombinant AO7. Excess AO7 did not compete the binding of RCBTB1 to UbcM2 (data not shown), and we conclude that RING-finger containing proteins do not compete with RCBTB1 for a common binding site on UbcM2.

To further map the domains mediating the RCBTB1−UbcM2 interaction, we tested and quantitated the binding of a series of ^{35}S-labeled RCBTB1 truncation mutants (Figure 1E) to recombinant myc-UbcM2-His_6. Nonspecific binding of the truncations was minimal (Figure 1E, lanes 7−12). These experiments revealed that, on average, (i) removal of the C-terminal 87 residues of RCBTB1 decreased UbcM2 binding by 63%, (ii) removal of the BTB domain and the C-terminal 87 residues decreased UbcM2 binding by 78%, and (iii) deletion of the BTB domain alone decreased binding by 55%. In addition, a fragment encompassing the isolated BTB domain and C-terminal 87 residues bound UbcM2 twice as well as full-length RCBTB1, and a fragment representing the C-terminal 87 residues of RCBTB1 bound comparably to wild type. This 87-residue fragment only has a single methionine and is difficult to visualize (Figure 1E, lane 6, asterisk). These data confirm that the C-terminal 87 residues of RCBTB1 are sufficient to mediate the interaction with UbcM2, but that the adjacent BTB domain contributes to and/or stabilizes the interaction. The finding that a RCBTB1 fragment lacking residues 1−359 has increased binding to UbcM2 indicates that one or more domains within the N-terminal 359 residues of RCBTB1 may negatively regulate the interaction.

Because RCBTB1 harbors a BTB domain, we next tested if the protein had the properties of a CUL3 substrate adaptor (*19, 21, 31*). RCBTB1 and CUL3 were coexpressed in human embryonic kidney 293T (HEK293T) cells to determine if the two proteins could be coprecipitated. The results from these experiments (Figure 2A) showed that an RCBTB1−CUL3 complex can form and be isolated from cell lysates. The isolation of a complex between the endogenous proteins was precluded by a failure of our α-RCBTB1 antisera to detect endogenous RCBTB1 in several cell lines (data not shown).

Biochemical and structural studies of cullin-based E3 ligases have demonstrated that substrate adaptors such as skp1/2, elongin B/C, and BTB domain proteins bind the N-terminal domains of their cognate cullins (*10, 19, 31, 39*).

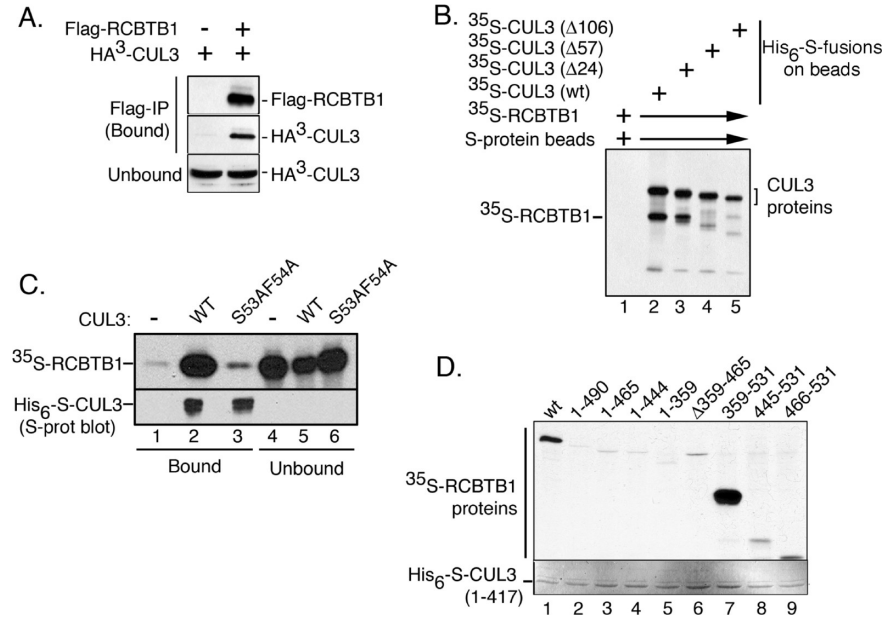

FIGURE 2: RCBTB1 has the biochemical properties of a CUL3 substrate adaptor. (A) A RCBTB1−CUL3 complex can be recovered from cell lysates. HEK293T cells were transfected with plasmids encoding triple HA-tagged CUL3 (HA^3-CUL3) (lane 1) alone or with Flag-tagged RCBTB1 (Flag-RCBTB1) (lane 2). Lysates were combined with Flag-agarose, and the bead-associated and unbound proteins were analyzed by Western blotting with anti-Flag and anti-HA antibodies. (B) Deletion of the N-terminal 57 residues of CUL3 blocks RCBTB1 binding. A set of His_6-S-tagged CUL3 proteins and RCBTB1 were expressed in separate TNT reactions. Each ^{35}S-labeled CUL3 protein was then combined with an equal aliquot of ^{35}S-labeled RCBTB1 and S-protein agarose beads. Bead-associated proteins were separated by SDS−PAGE and visualized by fluorography. The structural and functional integrity of the CUL3 truncations is demonstrated in Figure 3E. (C) ^{35}S-labeled RCBTB1 was expressed in a TNT reaction and combined with S-protein agarose (−), or beads plus either recombinant wild type (WT) or (S53A, F54A) CUL3 from bacteria. Aliquots of bound (lanes 1−3) and unbound proteins (lanes 4−6) were resolved by SDS−PAGE and visualized by fluorography (^{35}S-RCBTB1) or blotting with S-protein HRP (His_6-S-CUL3). (D) Recombinant His_6-S-CUL3 (1−417) was immobilized on S-protein beads and combined with the indicated ^{35}S-labeled RCBTB1 proteins. The precipitated ^{35}S-labeled proteins were resolved by SDS−PAGE and visualized by fluorography. Recombinant His_6-S-CUL3 (1−417) was detected by CBB staining. Nonspecific binding of the ^{35}S-labeled proteins to the S-protein agarose is shown in Supplemental Figure 1 in the Supporting Information.

This binding is mediated by a highly conserved hydrophobic helix (e.g., referred to as H2 helix) present in all cullins (e.g., refs *10*, *19*). In CUL3, helix 2 spans residues 51 to 67 (*31*). To determine if RCBTB1 interacts with CUL3 through helix 2, we generated a series of N-terminal truncations of CUL3 and assessed their capacity to coprecipitate ^{35}S-labeled RCBTB1. RCBTB1 was coprecipitated by wild type CUL3 and CUL3 (Δ24), a truncation lacking the N-terminal 24 residues (Figure 2B, lanes 2 and 3), but not by CUL3 (Δ57) and CUL3 (Δ106), mutants which lack an intact helix 2 (Figure 2B, lanes 4 and 5). As shown in Figure 3E, all of the CUL3 truncations folded properly as evidenced by their binding to Roc1.

We extended these findings by testing the binding of RCBTB1 to CUL3 (S53A, F54A), a double point mutant lacking the capacity to interact stably with substrate adaptors (*31, 40*). Wild type CUL3 coprecipitated RCBTB1 whereas the mutant did not (Figure 2C, top panel). We next tested if the BTB domain of RCBTB1 was required for the RCBTB1−CUL3 interaction using recombinant, His_6-S-tagged CUL3 (1−417) and a panel of ^{35}S-labeled RCBTB1 proteins. CUL3 (1−417) is sufficient for substrate adaptor binding (*19, 31*) and, as expected, bound wild type RCBTB1 but not to various carboxy-terminal truncations (Figure 2D, lanes 2−5). Deletion of the BTB domain from RCBTB1 also largely abrogated CUL3 binding (Figure 2D, lane 6). CUL3, however, bound robustly to a fragment of RCBTB1 encompassing the BTB domain and the carboxy-terminal tail (Figure 2D, lane 7). This same RCBTB1 fragment interacted with UbcM2 (Figure 1E, lane 5). In contrast, disruption or removal of the BTB domain from this fragment dramatically reduced binding to CUL3 (Figure 2D, lanes 8 and 9). Nonspecific binding of the various ^{35}S-labeled RCBTB1 proteins is shown in Supplemental Figure 1 in the Supporting Information. These data indicate that RCBTB1 interacts with CUL3 in a BTB domain-dependent fashion and that the 87-residue, carboxy-terminal tail of RCBTB1 contributes to the interaction. These results further support the conclusion that RCBTB1 has the properties of a CUL3 substrate adaptor.

The finding that a putative CUL3 substrate adaptor (RCBTB1) was a binding partner of UbcM2 indicated that the enzyme might engage CUL3 ligase complexes. This link was further strengthened by the finding that both UbcM2 and CUL3 bound the same domain of RCBTB1 (Figure 1E, lane 5, and Figure 2D, lane 7). To investigate the relationship between UbcM2 and CUL3, we did pulldown assays using GST-UbcM2 and ^{35}S-labeled CUL3 as we did not detect a direct interaction between bacterially expressed UbcM2 and CUL3 (data not shown). We found that wild type and catalytically inactive UbcM2 precipitated CUL3 (Figure 3A). Unexpectedly, we also found that a mutant enzyme, UbcM2 (F122A), which lacks the capacity to stably interact with RING-finger proteins (see Figures 1C and 1D), bound CUL3 (Figure 3A, lane 4). This result was surprising because the architecture of a prototypical CUL3-based ligase (Figure 3B) shows that E2s are recruited into the complex through a bridging RING-finger protein, namely, Roc1/Rbx/Hrt1 (*11, 12*).

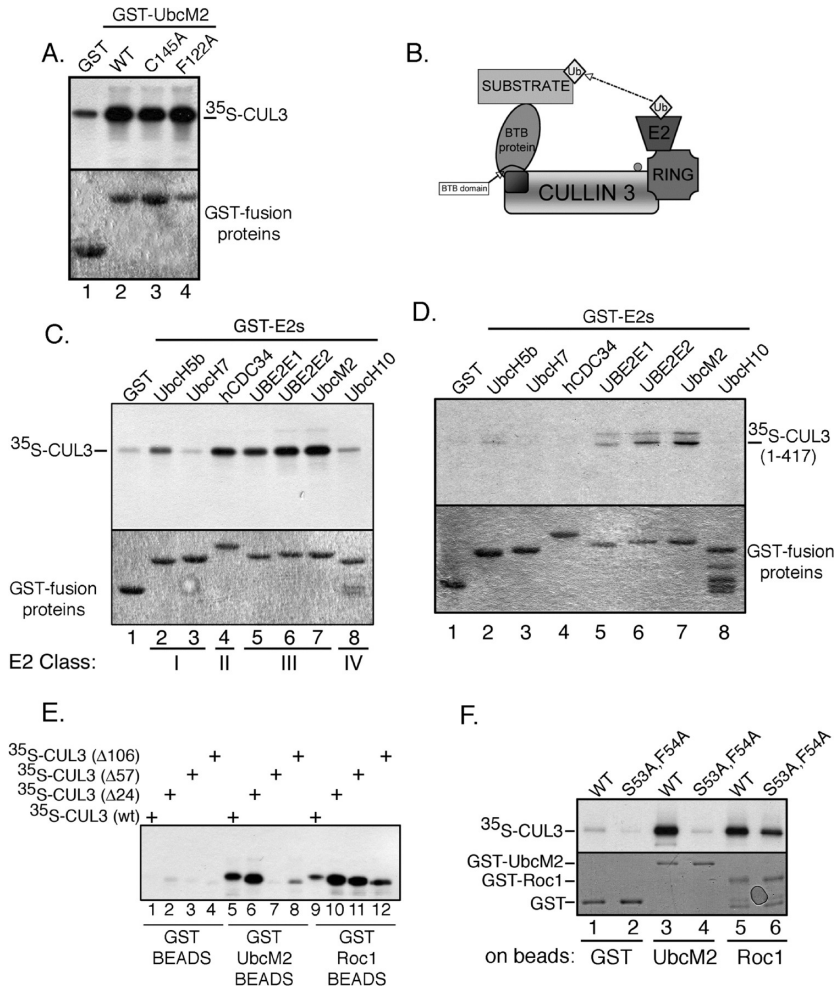

FIGURE 3: UbcM2 interacts with the domain of CUL3 that recruits substrate adaptors. (A) ^{35}S-labeled CUL3 was incubated with GSH-sepharose and either GST or the indicated GST-UbcM2 fusion proteins. Bead-bound proteins were resolved by SDS−PAGE. ^{35}S-labeled CUL3 was visualized by fluorography (top panel) and the GST proteins by CBB-staining (bottom panel). (B) Cartoon illustrating the architecture of a prototypical CUL3-based E3 ligase. CUL3 is bound at its N-terminal end to a BTB-containing substrate adaptor (BTB protein) and through its C-terminal domain to a RING-finger protein (RING). The BTB protein recruits substrates for ubiquitin modification and the RING-finger protein recruits an E2 into the complex. The E2 transfers ubiquitin (Ub) to the substrate. A conserved lysine in the C-terminal domain of CUL3 is modified with the ubiquitin-like modifier, Nedd8 (shown as a ball). (C) CUL3 can be precipitated by a select group of E2s. ^{35}S-labeled CUL3 was combined with GST (lane 1) or the indicated GST-E2 fusion proteins (lanes 2−8) and GSH-sepharose. The class to which each E2 belongs is indicated below the lane numbers. (D) Same experiment as (C) except ^{35}S-labeled CUL3 (1−417) was expressed in place of full length CUL3. (E) UbcM2 binding to CUL3 requires the N-terminal 57 residues of the cullin. A set of His_6-S-tagged CUL3 proteins was expressed in individual TNT reactions and combined with GSH-sepharose and either GST (lanes 1−4), GST-UbcM2 (lanes 5−8), or GST-Roc1 (lanes 9−12). (F) Wild type or S53A, F54A CUL3 were expressed in TNT reactions and combined with GSH-sepharose and either GST (lanes 1, 2), GST-UbcM2 (lanes 3, 4), or GST-Roc1 (lanes 5, 6). For the data shown in A, C, D, E, and F, bead-associated proteins were separated by SDS−PAGE and visualized by either fluorography (^{35}S-labeled proteins) or CBB staining (GST proteins).

To further investigate the specificity of the UbcM2−CUL3 interaction, we tested the capacity of different GST-E2 fusion proteins to precipitate CUL3. E2s are broadly grouped into 4 classes, and representatives from each were tested. All class III E2s (UbcM2, UBE2E1, and UBE2E2) bound CUL3 (Figure 3C, lanes 5−7), as did hCDC34, a class II E2 (Figure 3C, lane 4). UbcH5b, a class I E2, bound weakly to CUL3 (Figure 3C, lane 2). UbcH10 and UbcH7 did not bind the cullin (Figure 3C, lanes 3 and 8) above background (Figure 3C, lane 1). These data indicate that a subset of E2s selectively interacts with CUL3 in this assay.

Based on our data showing that UbcM2 does not require a bridging RING-finger protein to interact with CUL3 (Figure 3A, lane 4), we next tested if the class III E2s bind the N-terminal half of CUL3 (residues 1−417). This CUL3 fragment lacks the binding site for Roc1 (i.e., residues 597−615 (*19*)) but encompasses the domain that mediates substrate adaptor binding (*19, 21, 31*). Pulldowns were done with GST-E2 proteins and ^{35}S-labeled-CUL3 (1−417). UbcM2, UBE2E2, and to a lesser extent UBE2E1 bound the N-terminal half of CUL3 (Figure 3D, lanes 5−7) whereas the other E2s did not (Figure 3D, lanes 2 and 4).

We fine-mapped the interaction of UbcM2 within the N-terminal half of CUL3 using CUL3 truncations expressed in the TNT system. GST-UbcM2 bound wild type CUL3 and a truncation lacking the N-terminal 24 residues (Figure 3E, lanes 5 and 6), but the mutants bearing larger deletions were not appreciably precipitated (Figure 3E, lanes 7 and 8). All the truncations were functional as indicated by their interaction with GST-Roc1 (Figure 3E, lanes 9−12). These data corroborate our findings that UbcM2 associates with the N-terminal half of CUL3 (Figure 3D, lane 7). Interest-

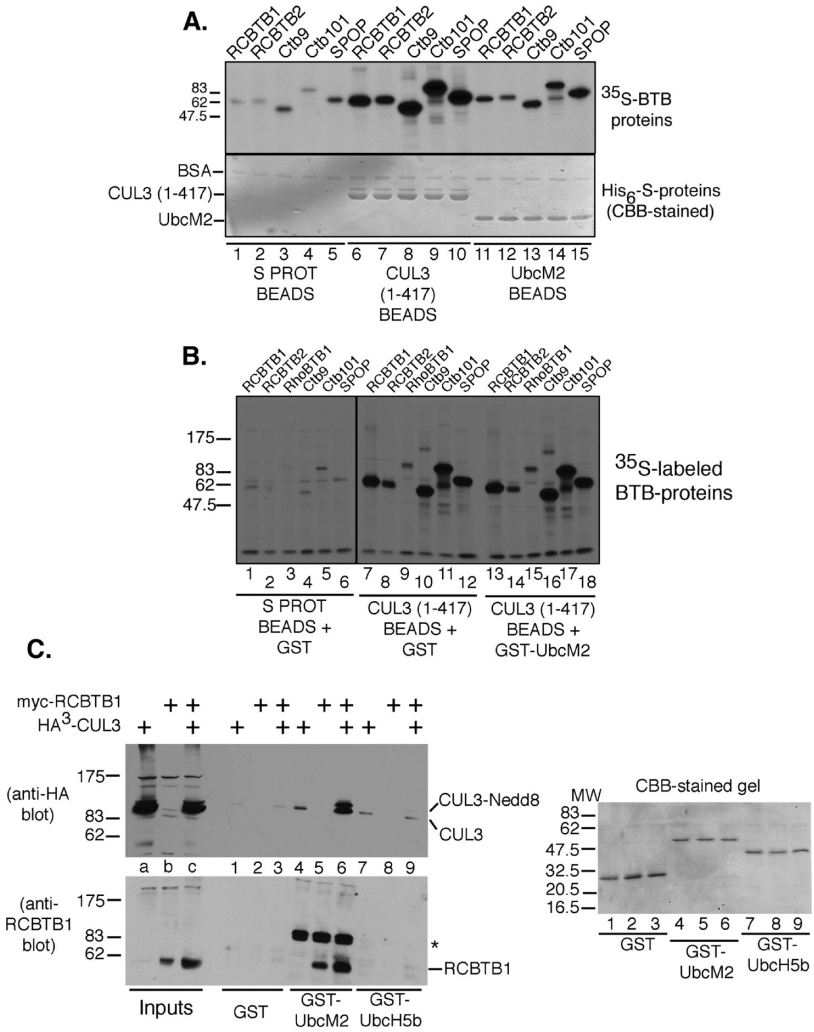

FIGURE 4: UbcM2 interacts with a panel of CUL3 substrate adaptors. (A) ^{35}S-labeled, BTB-containing substrate adaptors were combined with either control S-protein beads (lanes 1−5), or recombinant, immobilized His$_6$-S-CUL3 (1−417) (lanes 6−10), or recombinant, immobilized His$_6$-S-UbcM2. Bead-associated proteins were separated by SDS−PAGE and visualized by either fluorography (^{35}S-labeled proteins) or CBB staining (His$_6$-S-tagged proteins). (B) Similar experiment to (A) using His$_6$-S-CUL3 (1−417) on beads, and reaction mixtures were supplemented with 10 μM GST (lanes 1−12) or 10 μM GST-UbcM2 (lanes 13−18). For (A) and (B), the migration of molecular weight markers is indicated on the left. (C) Overexpressed RCBTB1 increases the binding of unmodified and neddylated CUL3 to UbcM2. HEK293T cells were transiently transfected with plasmids expressing myc-RCBTB1, HA^3-CUL3, or myc-RCBTB1 and HA^3-CUL3. Lysates derived from the transfected cells were combined with GSH-sepharose and either GST, GST-UbcM2, or GST-UbcH5b. Bead-associated proteins were analyzed by SDS−PAGE and Western blotting. Myc-RCBTB1 was detected with anti-RCBTB1, and HA^3-CUL3 was detected with anti-HA 12CA5. The right panel shows the GST proteins stained with CBB. Asterisk represents the cross-reactivity of the anti-RCBTB1 antiserum with GST-UbcM2 (bottom blot, lanes 4−6).

ingly, the domain of CUL3 that interacts with UbcM2 overlaps with the region bound by RCBTB1 (Figure 2B) and other BTB substrate adaptors (*19, 21, 31*).

The binding of UbcM2 and RCBTB1 to a common domain of CUL3 lead us to test if the interaction of UbcM2 with CUL3 was coupled to substrate adaptor recruitment. This was done by determining if UbcM2 could bind the double point mutant of CUL3 that fails to associate with substrate adaptors (e.g., Figure 2C). Wild type or (S53A, F54A) CUL3 were expressed in separate TNT reactions and combined with GST, GST-UbcM2, or GST-Roc1. GST-UbcM2 bound wild type CUL3 but not the mutant (Figure 3F). The mutant was not globally misfolded as demonstrated by its capacity to bind GST-Roc1. These data demonstrate that UbcM2 and substrate adaptors associate with a common domain of CUL3 and imply that the interaction of UbcM2 with CUL3 either requires the presence of a substrate adaptor or alternatively, is mutually exclusive with substrate adaptor binding.

We next tested if UbcM2 interacted with other BTB-containing substrate adaptors using recombinant His$_6$-S-UbcM2 and ^{35}S-labeled BTB proteins expressed in individual TNT reactions. The proteins tested were (i) RCBTB1, (ii) the closely related RCBTB2, (iii) Ctb9, (iv) Ctb101, and (v) SPOP (*41, 42*). To demonstrate that each BTB protein could bind CUL3, aliquots of the TNT reactions were also incubated with His$_6$-S-tagged CUL3 (1−417). These experiments demonstrated that both CUL3 (1−417) (Figure 4A, lanes 6−10) and UbcM2 (Figure 4A, lanes 11−15) specifically bound all of the BTB proteins. These findings imply that UbcM2 can engage multiple CUL3 ligase complexes and has the properties of a general cofactor.

Because UbcM2 and substrate adaptors associate with a common domain of CUL3, we next tested if the binding of UbcM2 to CUL3 influenced substrate adaptor docking. Using the same experimental approach shown in Figure 4A, binding reactions were supplemented with 10 μM GST (Figure 4B,

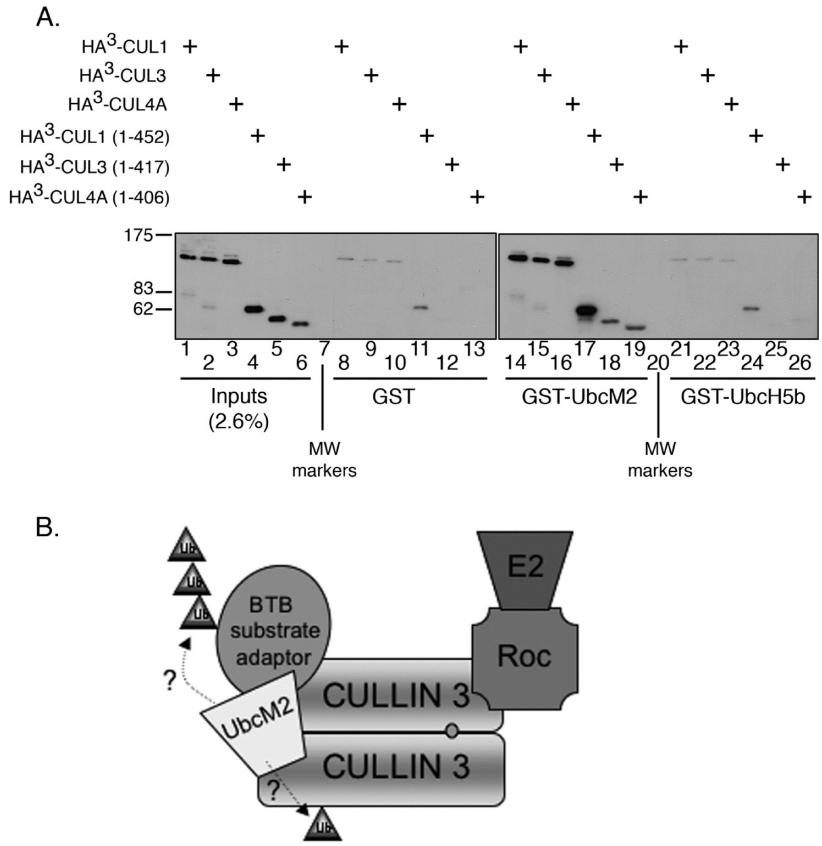

FIGURE 5: UbcM2 binds to the N-terminal halves of multiple cullins. (A) HEK293T cells were transfected with plasmids encoding the indicated HA^3-tagged cullins (full-length or N-terminal halves). Lysates from the transfected cells were combined with GSH-sepharose and either GST (lanes 8−13), GST-UbcM2 (lanes 14−19), or GST-UbcH5b (lanes 21−26). The bead-associated HA^3-tagged cullins were analyzed by anti-HA 12CA5 Western blotting. Molecular weight (MW) markers were run in lanes 7 and 20, and inputs for each pulldown are shown in lanes 1−6. (B) Model of UbcM2 association with activated CUL3 ligases. In contrast to the Roc1/Rbx1-dependent recruitment of E2s to the C-terminal end of CUL3 complexes, we propose that UbcM2 can be recruited, in concert with substrate adaptors, to the N-terminal half of activated CUL3. The function(s) of UbcM2 in this context remains to be determined, but we speculate that the enzyme may ubiquitylate the cullin and/or the substrate adaptors. The small ball between the dimerized cullin represents Nedd8. The substrate adaptor is shown as a monomer but may be a dimer in many cases (*49*).

lanes 1−12) or 10 μM GST-UbcM2 (Figure 4B, lanes 13−18) and the amount of bound BTB-domain proteins was analyzed. We found that a large excess of UbcM2 did not compete with the binding of substrate adaptors to CUL3 (Figure 4B, compare lanes 7−12 with 13−18). Thus, the interaction of UbcM2 with CUL3 is not mutually exclusive with substrate adaptor recruitment indicating that UbcM2 likely binds substrate adaptor-loaded CUL3 complexes.

Additional support for this interpretation came from an experiment testing if substrate adaptor coexpression influenced the amount of CUL3 precipitated by UbcM2. RCBTB1 and CUL3 were overexpressed alone or together in HEK293T cells and the transfected-cell lysates were used for pulldowns with GST, GST-UbcM2, or GST-UbcH5b. UbcM2 precipitated RCBTB1 (Figure 4C, bottom panel) and CUL3 (Figure 4C, top panel) when each was expressed individually. Further, when RCBTB1 and CUL3 were coexpressed, UbcM2 precipitated a greater amount of CUL3 (Figure 4C). Interestingly, the CUL3 precipitated from the coexpression lysate showed a nearly 1:1 ratio of neddylated cullin to unmodified cullin. Overexpression of SPOP gave the same results (data not shown). The identification of the slower migrating band as neddylated CUL3 was demonstrated using green fluorescent protein (GFP)-tagged Nedd8 (data not shown). These data reveal that UbcM2 associates with both unmodified and neddylated CUL3, a heterodimer of which represents the activated form of CUL3 ligases (*20*). In contrast, UbcH5b showed no capacity to precipitate RCBTB1 and minimal capacity to stably associate with CUL3 (Figure 4C). These results are consistent with UbcM2 and substrate adaptors associating simultaneously with CUL3, suggesting that UbcM2 is recruited to activated CUL3 ligases.

Lastly, we tested if UbcM2 might be a general cofactor for multiple cullins. We used CUL1, CUL3, and CUL4A for these experiments. Each cullin was overexpressed in HEK293T cells as either a full-length protein or as a fragment representing the N-terminal half of the cullin. Transfected-cell lysates were then precipitated with either GST, GST-UbcM2, or GST-UbcH5b. Neither GST nor GST-UbcH5b (Figure 5A) precipitated the cullins to an appreciable extent. In contrast, GST-UbcM2 bound all three full-length cullins and their respective N-terminal halves (Figure 5A). These results bolster the notion that UbcM2 is a general cofactor for cullin-based E3 ligases.

DISCUSSION

In the work reported here, we have made two important discoveries. First, we have established through biochemical characterization that the disease-associated protein, RCBTB1, is a putative substrate adaptor for CUL3. Second, we have identified novel interactions between the highly conserved

E2, UbcM2, and components of cullin ligases. These interactions center around the association of UbcM2 with the substrate adaptor recruitment domain of CUL3 and imply that UbcM2 (and likely all class III E2s) regulates cullin-based ligases by a novel mechanism.

RCBTB1 is a well-conserved protein also known as CLLD7. The gene encoding RCBTB1 maps to chromosome band 13q14.3, an area frequently deleted in B-cell chronic lymphocytic leukemia and other lymphocytic neoplasias (*32*). Despite its potential clinical importance, the function of RCBTB1 is unknown. The protein reportedly can influence the capacity of the angiotensin II type 1A receptor to induce cellular hypertrophy (*43*). However, the mechanism underlying this effect has not been determined. The presence of a BTB domain in RCBTB1 predicted that the protein could be a substrate adaptor for a CUL3-based E3 ligase (e.g., refs *31*, *44*−*46*). Our data support this notion. We have demonstrated that RCBTB1 forms a complex with CUL3 in cell lysates (Figure 2A), binds the substrate-adaptor binding domain of CUL3 (Figure 2B), and fails to bind a CUL3 mutant deficient in substrate adaptor binding (Figure 2C) (*31*). In addition, we show that deletion of the BTB domain from RCBTB1 largely abrogates binding to CUL3 (Figure 2D). By characterizing RCBTB1 as a CUL3 substrate adaptor, our data predict that the loss of RCBTB1 in hematological malignancies results in the deleterious accumulation of one or more substrates. Thus, the identification of RCBTB1 substrates could have direct clinical implications.

Definitive evidence that RCBTB1 is a *bona fide* substrate adaptor requires the identification of a substrate(s) for a RCBTB1−CUL3 ligase. Toward this end, we carried out three independent yeast two-hybrid screens to identify putative RCBTB1 substrates. One of the screens retrieved three independent overlapping clones of the closely related RCBTB2 protein. The other two screens did not yield any verifiable interactions. We validated the interaction between RCBTB1 and RCBTB2 using pulldown (Supplemental Figure 2A in the Supporting Information) and colocalization studies (Supplemental Figure 2B in the Supporting Information). The pulldown studies demonstrated that RCBTB1 and RCBTB2 hetero- and homodimerize, a characteristic common to many CUL3 substrate adaptors (*47*−*51*). Similar to the model put forth by Sumara et al. regarding the cooperation of KLHL9 and KLHL13 heterodimers to target Aurora B kinase for CUL3-mediated degradation (*51*), RCBTB1 and RCBTB2 heterodimers may well constitute the functional CUL3 substrate adaptor. This could explain the failure to retrieve candidate substrates from multiple yeast two-hybrid screens using only RCBTB1 as the bait. We also tested whether the angiotensin II receptor or RCBTB2 were substrates for a RCBTB1−CUL3 ligase, but the degradation of neither protein was detectably influenced by RCBTB1 overexpression (data not shown).

In addition to providing evidence consistent with RCBTB1 being a CUL3 substrate adaptor, our identification of RCBTB1 as a binding partner of UbcM2 led to a series of findings which can be reconciled by a novel model for the association of UbcM2 with CUL3 ligases (Figure 5B). We propose that UbcM2 (and likely the other class III E2s (Figure 3D)) can be recruited, in concert with BTB substrate adaptors, to activated CUL3 ligases. This model is supported by multiple lines of evidence. First, UbcM2 and substrate adaptors bind the same domain of CUL3 (Figures 2B and 3E) and these interactions are not mutually exclusive (Figure 4B). Second, overexpression of different substrate adaptors increases the amount of CUL3 precipitated by UbcM2 (e.g., Figure 4C and data not shown). Third, a mutant of CUL3 that cannot recruit substrate adaptors (Figure 2C) also does not recruit UbcM2 (Figure 3F). And fourth, UbcM2 can interact with multiple substrate adaptors (Figure 4A) and this binding is largely mediated by the BTB domain (Figure 1E). A reasonable prediction of this model is that UbcM2 should efficiently bind a Nedd8-modified CUL3 heterodimer since this form of the cullin is actively engaged with substrate adaptors and their cognate substrates (*20*). Consistent with this prediction, UbcM2 precipitated an equal ratio of unmodified and Nedd8-bound CUL3 from cell lysates in which RCBTB1 (Figure 4C) or SPOP (not shown) were overexpressed. We suspect that UbcM2 may similarly engage other cullin-based ligases considering: (i) the enzyme bound the amino terminal domains of CUL1 and CUL4A (Figure 5A), (ii) the structural similarity between BTB domains, Skp1, and the CUL2 adaptor, Elongin C (*9*), and (iii) the class III E2s have been recovered in pulldowns from cells with the CUL4A substrate adaptor, de-etiolated 1 (*36*).

Although we do not yet know how UbcM2 regulates CUL3 ligase activity, one possibility is that the enzyme modulates the conformation of CUL3 by ubiquitylation. This notion is based on our finding that UbcM2 can monoubiquitylate CUL3 *in vitro* (Supplemental Figure 3 in the Supporting Information) and by recent data describing the conformational changes to cullins induced by Nedd8 attachment (*7*). The recruitment of UbcM2 to substrate adaptor-loaded CUL3 may also provide a means for ubiquitylating substrate adaptors thus enabling CUL3 to efficiently switch between substrate adapters and thus substrates. Although we did not observe this UbcM2-induced autoubiquitylation of RCBTB1 (Plafker and Plafker, unpublished results), it is conceivable that, under different experimental conditions or using a different substrate adaptor, such effects of the enzyme could be revealed.

In summary, we have identified RCBTB1 as a substrate adaptor for CUL3, strengthening the link between malfunction of the ubiquitin proteolytic system and the etiology of hematological malignancies (*52*). We also have identified and characterized a unique feature of the class III E2s. These enzymes were originally cloned by Matuschewski and colleagues and distinguished for the presence of N-terminal extensions (*53*). More recently, we reported that these enzymes are distinguished from other E2s by binding to the transport receptor, importin-11 (*54*), and by being imported into the nucleus in an activation-dependent fashion (*33*). We have now found that these enzymes are further distinguished by a unique capacity to engage cullin complexes, in agreement with their recovery from pulldowns using CUL4A substrate adaptors (*36*). Perhaps the most remarkable aspect of this association with cullins and their substrate adaptors is that it is independent of the critical residues utilized by E2s in typical E3 binding interactions. That is, it does not require the highly conserved phenylalanine, proline, and alanine present in most E2s that enable binding to both RING-finger domains and HECT domains (*37, 38*). This new paradigm expands the repertoire of interactions and functions

that E2s can participate in and positions the class III E2s as novel regulators of cullin-based ligases.

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SUPPORTING INFORMATION AVAILABLE

Supplemental Figure 1 shows fluorographs of inputs and nonspecific association of ^{35}S-RCBTB1 proteins with S-protein agarose. These controls accompany Figures 1E and 2D. Supplemental Figure 2 shows data supporting the conclusion that RCBTB1 and the closely related protein, RCBTB2, can homo- and heterodimerize. Supplemental Figure 3 shows data demonstrating that UbcM2 can monoubiquitylate CUL3 in vitro and that this ubiquitylation is independent of a bridging RING-finger protein or an intact site for Nedd8 attachment on CUL3. This material is available free of charge via the Internet at http://pubs.acs.org.

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