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Targeting of protein ubiquitination by BTB–Cullin 3–Roc1 ubiquitin ligases

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The concentrations and functions of many cellular proteins are regulated by the ubiquitin pathway. Cullin family proteins bind with the RING-finger protein Roc1 to recruit the ubiquitin-conjugating enzyme (E2) to the ubiquitin ligase complex (E3). Cul1 and Cul7, but not other cullins, bind to an adaptor protein, Skp1. Cul1 associates with one of many F-box proteins through Skp1 to assemble various SCF–Roc1 E3 ligases that each selectively ubiquitinate one or more specific substrates. Here, we show that Cul3, but not other cullins, binds directly to multiple BTB domains through a conserved amino-terminal domain. *In vitro*, Cul3 promoted ubiquitination of *Caenorhabditis elegans* MEI-1, a katanin-like protein whose degradation requires the function of both Cul3 and BTB protein MEL-26. We suggest that *in vivo* there exists a potentially large number of BCR3 (BTB–Cul3–Roc1) E3 ubiquitin ligases.

Ubiquitination requires a cascade of three enzymatic activities for activating (E1), conjugating (E2) and ligating (E3) ubiquitin covalently to a substrate. The E3 ubiquitin ligases provide two distinct functions: catalysing isopeptide bond formation and targeting of the substrate[1–3]. Two major families of E3 ligases have been described: the HECT-domain family that is defined by its homology to E6-associated protein (E6AP) carboxyl terminus and the RING family that contains either an intrinsic RING-finger domain or an associated RING-finger protein subunit essential for ubiquitin ligase activity[4–6].

One of the best-characterized RING E3 ligases is the SKP1–cullin1–F box–Roc1 protein (SCF–Roc1) complex, in which Cul1 binds to an adaptor molecule, SKP1, through an N-terminal domain and with a small RING-finger protein, Roc1 (RING of cullins, also known as Hrt1 and Rbx1), through a carboxy-terminal domain[7]. Through Skp1, Cul1 associates with an F-box protein that in turn binds a phosphorylated substrate[8–10]. The Cul1 component of the SCF E3 ligase belongs to an evolutionarily conserved family of proteins known as cullins, of which there are six closely related members (Cul1, 2, 3, 4A, 4B and 5)[11] and three distant relatives (Cul7, Parc and APC2) in mammalian cells. In a somewhat similar arrangement to SCF–Roc1, human Cul2 binds directly to Elongin BC and indirectly to a specificity determinant VHL (von Hippel-Lindau) to assemble CBC^{VHL}–Roc1 ligase to ubiquitinate substrates such as HIF-1α (hypoxia-inducible factor 1α)[12–16]. Through their association with Roc1 or Roc2, all cullins constitute active ubiquitin ligases[17], but only Cul1 and Cul7 interact with Skp1 (refs 18–20). Here, we wanted to address how other cullins target their substrates.

Our initial efforts to determine the substrates and substrate-targeting mechanisms of Cul3 were focused on immunopurification of endogenous human Cul3 complexes. A large number of putative Cul3-interacting proteins were identified that were competed off after pre-incubation of the Cul3 antibody with a molar excess of antigen peptide (Fig. 1a). To strengthen the specificity of identifying Cul3-interacting proteins and to increase the chance of identifying substrates or protein(s) involved in substrate recruitment, we ectopically expressed and immunopurified Flag–Cul3 complexes, as well as complexes of Flag–Cul3$^{\Delta N41}$, which contains an internal deletion of 41 amino acids from Cul3 (from Trp 34 to Tyr 74; Fig. 1b). These 41 residues include a hydrophobic helix corresponding to the H2 helix in Cul1, which is involved in binding to Skp1 (ref. 7) and is highly conserved in cullin orthologues, but not in paralogues. We reasoned that a protein identified by two different antibodies, one recognizing Cul3 and one recognizing the Flag epitope fused with ectopically expressed Flag–Cul3, but not found in the Cul3$^{\Delta N41}$ complex, would most probably be a *bona fide* Cul3-interacting protein, as either a substrate or a protein involved in substrate-recruitment. More than 20 proteins were identified by mass spectrometric analysis from these purifications, including Cul3, neddylated Cul3 and subunits of the COP9 signalosome. Many proteins identified were not previously reported to interact with Cul3. These include two closely related proteins, KIAA1309 and KIAA1354, which were readily detected in both endogenous Cul3 and Flag–Cul3 immunocomplexes, but not in the Flag–Cul3$^{\Delta N41}$ complex, and were therefore chosen for detailed characterisation.

KIAA1309 and KIAA1354 encode polypeptides of 613 and 617 residues, respectively, share 89% identity over their entire sequences, and contain a BTB (Broad-complex, tramtrack and bric-a-brac) motif and six Kelch repeats. Coupled transfection and reciprocal immunoprecipitation–western blot analyses confirmed that both KIAA1309 and KIAA1354 bind efficiently to Cul3 *in vivo* (Fig. 1c). Under the same assay conditions, none of four other human cullins — Cul1, Cul2, Cul4A or Cul5 — were found to interact with either KIAA1309

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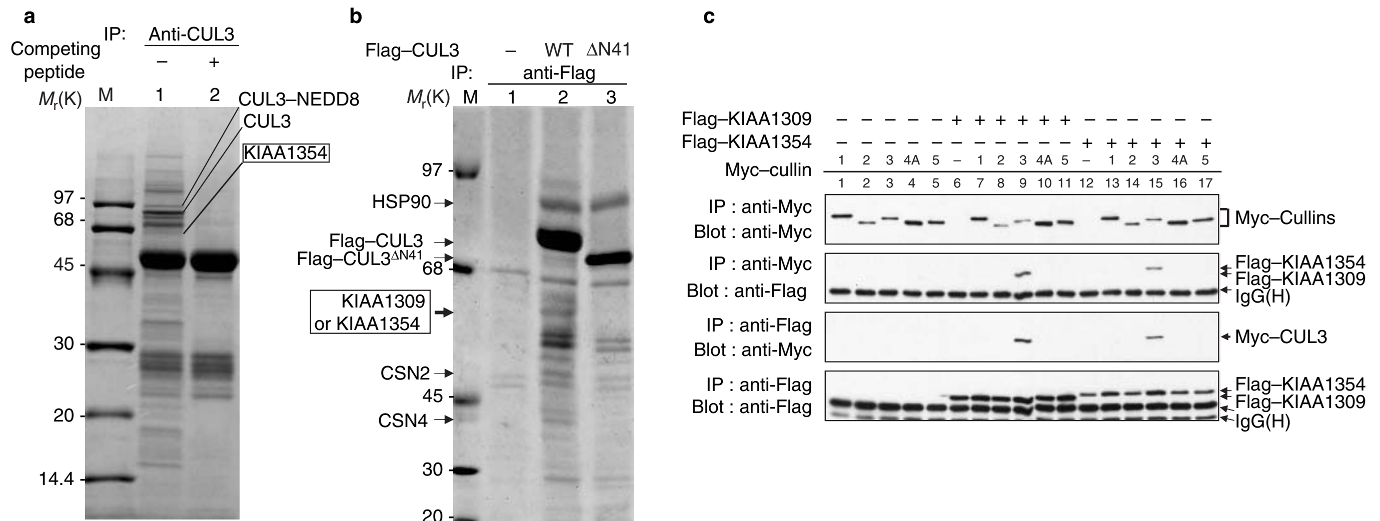

Figure 1 Purification of CUL3 complexes. (**a**) Clarified lysates derived from 293T cells were immunoprecipitated with an anti-Cul3 antibody, resolved by SDS–PAGE and stained with Coomassie blue. Specific Cul3-interacting proteins were identified by being competed off after addition of a molar excess of antigen peptide. The identities of proteins determined by mass spectrometric analysis are indicated. Seven peptides derived from a protein with relative molecular mass (M_r) of 65,000 identified by mass spectrometry match perfectly with KIAA1354. (**b**) Lysates derived from transfected 293T cells were precipitated with an anti-Flag antibody. Flag–Cul3 complexes were eluted with a Flag peptide, resolved by SDS–PAGE and stained with Coomassie blue. One peptide derived from a 65K protein identified by tandem mass spectrometry matches perfectly with both KIAA1354 and KIAA1309. (**c**) Plasmids expressing various human cullins were co-transfected into 293T cells with either KIAA1309 or KIAA1354. The cullin–KIAA1309 and KIAA1354 association was then examined by immunoprecipitation and western blotting.

or KIAA1354, indicating that KIAA1309 and KIAA1354 interact selectively with Cul3.

A series of small, domain-specific internal deletion mutants of Cul3 were generated and tested for binding to KIAA1309 (Fig. 2a). Deletions targeting the conserved central domain implicated in binding to the COP9 signalosome ($Cul3^{\Delta 24}$, deleting Leu 418–Leu 441; J. Lui and Y. X., unpublished observations), the Roc1-binding domain ($Cul3^{\Delta Roc1}$, deleting Phe 597–Glu 615), or the C-terminal sequence required for Nedd8 modification ($Cul3^{\Delta C21}$, deleting Leu 748–Ala 768) did not appreciably affect the binding of Cul3 to KIAA1309 (Fig. 2b). As expected, deletion of the N-terminal 41 residues completely disrupted binding of Cul3 to KIAA1309. However, the N-terminal 280 residues ($Cul3^{N280}$) were capable of binding to KIAA1309, albeit at lower levels than wild-type Cul3. Bacterially purified His-tagged $Cul3^{N197}$ was able to bind to similarly purified glutathione *S*-transferase (GST)–KIAA1309 (Fig. 2c). Together, these results indicate that the N-terminal domain of Cul3 is both necessary and sufficient for binding to KIAA1309 and KIAA1354, and that Cul3–KIAA1309 and Cul3–KIAA1354 association does not require additional adaptor molecule(s).

The BTB domain[21,22], also known as POZ (poxvirus and zinc finger)[23], and the Kelch domain[24] are both involved in mediating protein–protein interactions. A series of deletion mutants was generated in KIAA1309 to map the Cul3-binding site (Fig. 2d). Deletion of all six Kelch repeats ($KIAA1309^{N285}$) had no significant effect on Cul3–KIAA1309 binding, indicating that the Kelch repeats are not involved in binding to Cul3. Deletion of the N-terminal 30 residues ($KIAA1309^{\Delta N30}$) partially reduced Cul3 binding, whereas deletion of 150 residues from the N-terminus ($KIAA1309^{\Delta N150}$) completely abolished Cul3 binding (Fig. 2e). Hence, the BTB domain, not the Kelch repeats, binds to Cul3, a conclusion that was further supported by the binding of bacterially purified Cul3 with another BTB protein, BTBD1, which does not contain a Kelch repeat (Fig. 2c). Ectopically expressed KIAA1309 bound to endogenous Cul3, as well as Roc1, and deletion of the N-terminal BTB domain from KIAA1309 abolished the binding of both (Fig. 2f), indicating that KIAA1309 can form a ternary complex with Roc1 through Cul3.

The BTB domain is found in a large number of otherwise diverse proteins. We tested whether Cul3 could also interact with the BTB domain from other proteins in a yeast two-hybrid screen and a direct binding assay. Screening of a HeLa cDNA library using $Cul3^{N197}$ as bait identified more than a dozen positive clones (data not shown), including three BTB proteins: first, another BTB kelch protein (AB026190); second, SPOP (speckle-type POZ protein), which also contains a MATH domain (meprin and TRAF-C homology); third, the transcriptional repressor GCL (germ cell-less), which has no additional recognisable domain. When co-expressed with Cul3 in cultured 293T cells, the BTB domain from these three proteins readily bound to Cul3 (Fig. 3a). Separately, BTB domains from seven randomly selected BTB proteins were amplified and ectopically expressed, and their binding to Cul3 was assayed. All bound appreciably to Cul3 (Fig. 3a). Under the same binding assay conditions, Skp1, which is structurally related to BTB and binds an analogous N-terminal region in Cul1, did not exhibit any detectable binding to Cul3. Thus, together with KIAA1309, KIAA1354, BTBD1 and MEL-26 (see below), we have demonstrated binding of Cul3 to 13 distinct BTB domains (Fig. 3b), indicating that Cul3 could potentially interact with multiple BTB proteins *in vivo*.

Loss-of-function of the BTB protein MEL-26 and Cul3 in *C. elegans* phenocopy each other and stabilize MEI-1, which encodes a catalytic AAA ATPase and forms a katanin-like heterodimer with MEI-2 (refs 25–28). To test the possibility that Cul3 interacts with *C. elegans* MEL-26 and targets MEI-1 for ubiquitination, we first examined the binding of Cul3 and MEL-26. *In-vitro*-translated and radiolabelled *C.elegans* MEL-26 and Cul3 formed a complex, as determined by reciprocal immunoprecipitation (Fig. 4a). Similarly, human Cul3 bound to *C. elegans* MEL-26 when both proteins were ectopically expressed in

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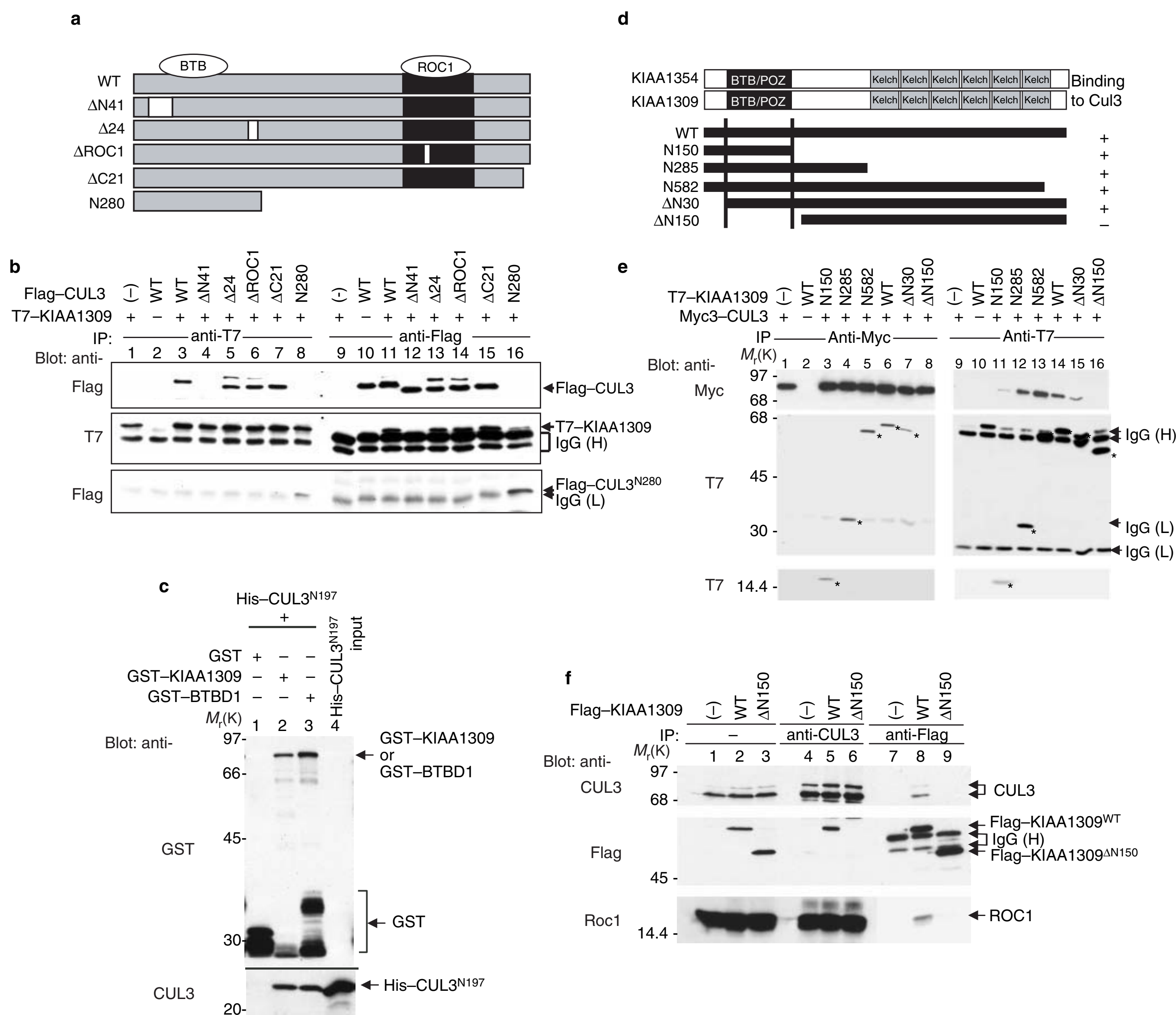

Figure 2 BTB domain of KIAA1309 binds to an N-terminal sequence of Cul3. (**a**) A schematic representation of wild-type human Cul3 and various domain-specific Cul3 mutants. (**b**) KIAA1309 was co-expressed with wild-type and various mutants of Cul3 in 293T cells, and binding was examined by immunoprecipitation and western blotting. (**c**) His–Cul3^{N197} fusion protein was purified from bacteria and mixed with GST alone, GST–KIAA1309 or GST–BTBD1 proteins. Mixtures were precipitated by glutathione beads and binding was examined by immunoblotting. (**d**) A schematic representation of wild-type and various deletion mutants of the BTB protein KIAA1309. (**e**) Myc3–Cul3 was co-expressed with wild-type and various mutants of KIAA1309 (denoted by an asterisk) in 293T cells, and binding was examined by immunoprecipitation and western blotting. (**f**) Complex formation between ectopically expressed KIAA1309 and endogenous Cul3 and Roc1 was determined by immunoprecipitation and western blotting.

cultured 293T cells (Fig. 4b). Cul3–MEL26 binding was almost completely disrupted by deletion of the N-terminal 41 residues from Cul3 required for binding to BTB domains, but not by deletion of the Roc1-binding site (Fig. 4b). We also demonstrated that *C. elegans* MEL-26, MEI-1 and MEI-2 formed a readily detectable ternary complex (Fig. 4c), providing biochemical evidence to suggest that MEL-26 interacts directly with MEI-1.

Next, we examined the interaction between Cul3 and MEI-1–MEL-26. Co-expression of Cul3 with MEL-26 and MEI-1 yielded a barely detectable Cul3–MEI1 complex (Fig. 4d). We reasoned that an active ubiquitin ligase and its substrate would not form a stable complex *in vivo*, therefore we examined the association of MEI-1with a Cul3 mutant deficient in Roc1 binding. Deletion of the Roc1-binding site from Cul3 was confirmed by metabolic radiolabelling–immunoprecipitation (see Supplementary Information, Fig. S1a), coupled immunoprecipitation–western blotting (data not shown), and by the substantial reduction of Cul3 auto-ubiquitin ligase activity (see Supplementary Information, Fig. S1b). Disruption of Roc1 binding considerably increased the association of Cul3 with MEI-1 (Fig. 4d). No discernible difference was detected in binding between MEL-26 and Cul3 when Roc1–Cul3 binding was disrupted (Fig. 4b). Thus, an inhibition of Cul3–Roc1 ligase activity stabilizes the Cul3–MEI-1 association.

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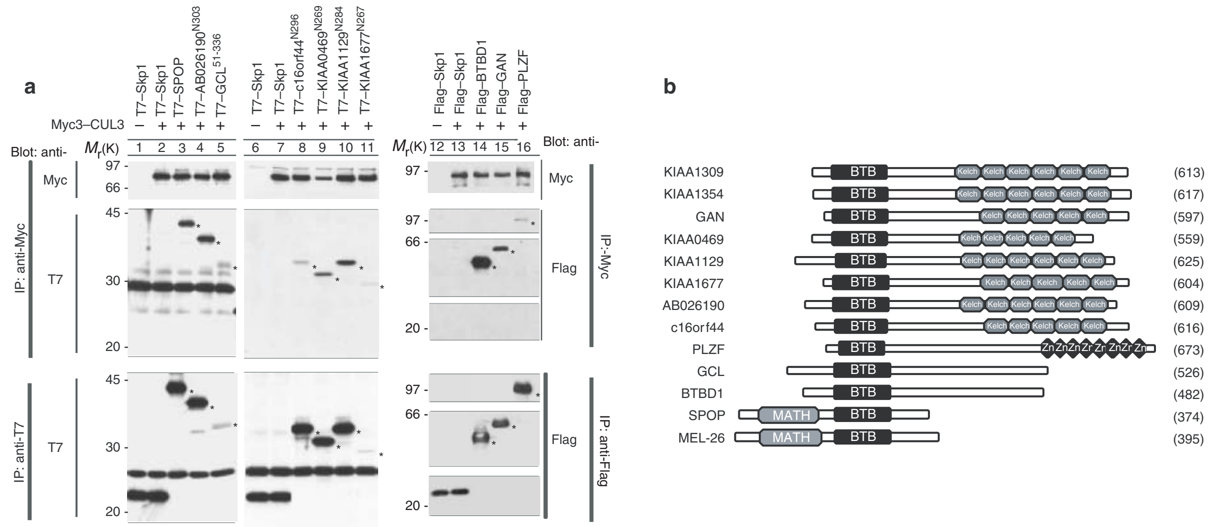

Figure 3 Cul3 binds to multiple BTB domains. (**a**) BTB domains from various proteins were amplified by PCR and ectopically expressed in 293T cells. Binding to Cul3 was assayed by immunoprecipitation and western blotting. Asterisks mark the expressed BTB polypeptides. (**b**) Schematic representation of 13 BTB proteins, whose binding with Cul3 has been experimentally demonstrated. Additional protein motifs present in these proteins are indicated. Numbers in parentheses denote the number of amino-acid residues of each protein.

To examine directly whether the Cul3–Roc1 ligase can promote MEI-1 ubiquitination *in vitro*, we immunoprecipitated the MEL26–Cul3–Roc1 complex from transfected 293T cells and used it as a source of E3 ligase. Association of Roc1 and MEL-26 with Cul3 was confirmed by co-immunoprecipiation (Fig. 5a, right). Incubation of the MEL26–Cul3–Roc1 immunocomplex with purified MEI1–MEI2 *in vitro* resulted in formation of characteristic incremental ubiquitin ladders (Fig. 5a). Omission of the MEL26–Cul3–Roc1 complex, ubiquitin or E2–Ubc5 abolished formation of the Flag–MEI-1 polyubiquitin ladder. Incubation of MEL26–Cul3–Roc1 with Flag–MEI-1 alone (that is, in the absence of Flag–MEI-2), gave rise to a similar MEI-1 polyubiquitin ladder, suggesting that at least under these *in vitro* assay conditions, MEI-2 is not required for ubiquitination of MEI-1. This is consistent with the observation that MEI-1 can associate with MEL-26 and Cul3 without co-expression of MEI-2 (Fig. 4b). Re-blotting the same filter with an anti-haemagglutinin (HA) antibody did not detect any HA–MEI-2 smear (data not shown), suggesting that under the same assay conditions, MEI-2 was either a poor substrate for, or cannot be ubiquitinated by, the MEL26-Cul3–Roc1 ligase. Disruption of either BTB- or Roc1-binding substantially decreased MEI-1 ubiquitination (Fig. 5b), supporting the idea that Cul3-mediated MEI-1 ubiquitination requires both MEL-26 and Roc1.

Finally, to further examine Cul3-mediated MEI-1 ubiquitination, we incubated bacterially purified MEI-1 with Cul3–Roc1 complex purified from insect cells. The amounts and purity of each purified protein was determined by SDS–PAGE (Fig. 5c, right), and the activity of the Cul3–Roc1 complex was determined by assaying for auto-ubiquitination activity (middle panel). After an *in vitro* ubiquitination reaction, MEI-1 was immunoprecipitated, resolved by SDS–PAGE and analysed by immunoblotting with antibodies against epitope-tagged ubiquitin (Fig. 5c, left). A high-molecular-weight ladder characteristic of a polyubiquitin chain was detected with two different antibodies recognizing HA- and Flag-tagged ubiquitin. Omitting E2, ubiquitin or MEI-1 abolished formation of the polyubiquitin ladder, confirming that the product was polyubiquitinated MEI-1. More importantly, omission of either Cul3–Roc1 or MEL-26 completely abolished MEI-1 polyubiquitination, suggesting that ubiquitination of MEI-1 is dependent on MEL26–Cul3–Roc1.

An important implication derived from SCF assembly is that through an adaptor protein, Skp1, the N-terminal region of Cul1 can interact with one of many F-box proteins present in the cell[9,10], thereby constituting various distinct SCF ligases and potentially targeting a large number of different substrates for ubiquitination. In this study, we provide evidence that Cul3 uses an N-terminal sequence conserved in Cul3 orthologues from different organisms, but not cullin paralogues, to interact with multiple BTB proteins. Furthermore, we demonstrate a direct interaction between *C. elegans* BTB protein MEL-26 and Cul3, and MEL26–Cul3–Roc1-dependent ubiquitination of MEI-1. We suggest that these Cul3-dependent ubiquitin ligases be described as BCR3, for BTB-Cul3-Roc, with a superscript to indicate the specific BTB protein (for example, $BCR3^{MEL-26}$). A notable difference between Cul1-mediated SCF and Cul3-mediated BCR3 ligases is the lack of a Skp1 adaptor in BCR3 (Fig. 2c).

A large number of BTB proteins exist in eukaryotes: 205 in human, 129 in *Drosophila melanogaster*, 103 in *C. elegans*, and 50 in *Arabidopsis thaliana* in the current database (http://www.sanger.ac.uk/Software/Pfam). Various physiological functions have been linked with different BTB proteins, ranging from developmental control to oncogenesis[29]. Our results provide the first clue (that is, recruiting specific proteins to Cul3–Roc1 ubiquitin ligase) of the biochemical function of the BTB domain. Structurally, BTB proteins can be categorized into four subgroups depending on the presence of C_2H_2 zinc fingers, Kelch repeats, MATH motifs and the BTB domain as the only recognizable motif. We have demonstrated the binding of Cul3 with at least one example from each subgroup. These findings suggest that Cul3–Roc1 could potentially constitute a large family of distinct BCR3 ubiquitin ligases with individual BTB proteins and target ubiquitination of many substrate proteins. □

METHODS

Immunopurification and mass spectrometric analysis of Cul3 complexes. To purify the endogenous Cul3 complex, 293T cells from five 150-mm plates were

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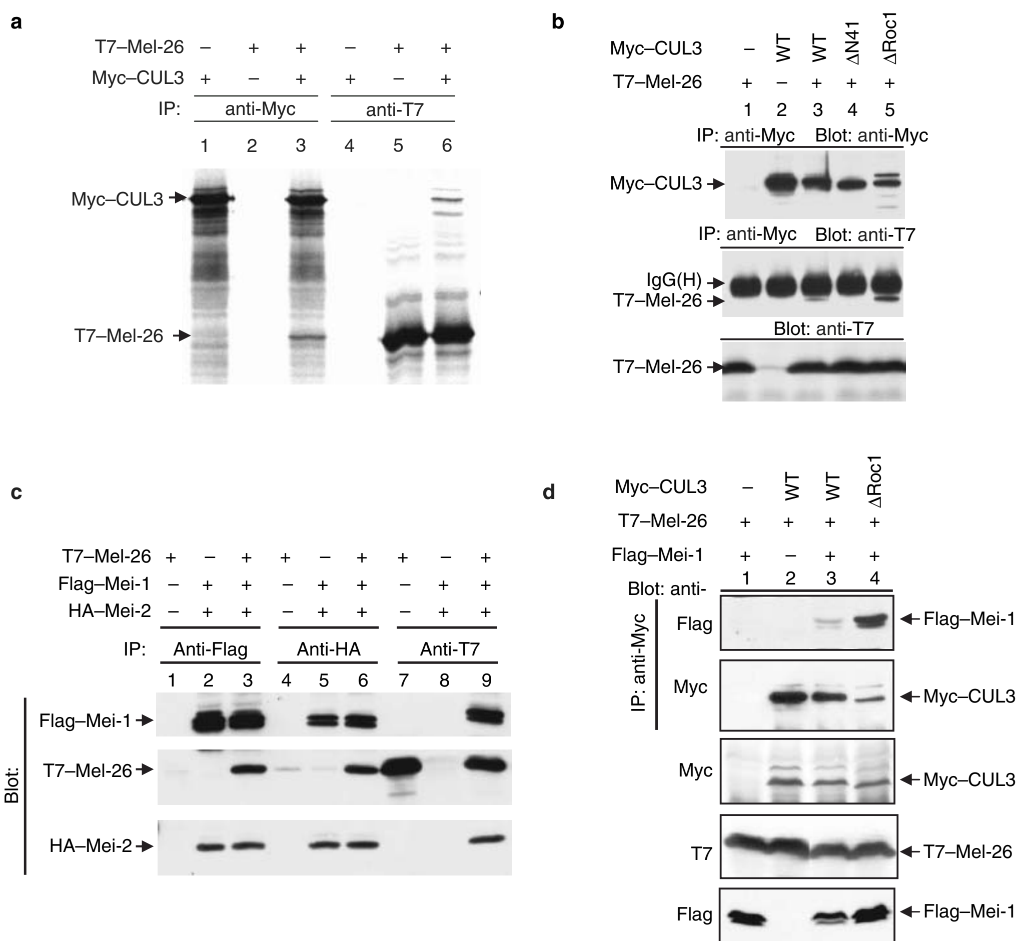

Figure 4 Cul3 binds to MEL-26 and MEI-1. (**a**) *In-vitro*-translated and radiolabelled *C. elegans* Cul3 and MEL-26 proteins were immunoprecipitated, resolved by SDS–PAGE and visualized by autoradiography. (**b**) Human Cul3 and *C. elegans* MEL-26 were co-expressed in human 293T cells and their association was examined by immunoprecipitation and western blotting. (**c**) *C. elegans* MEL-26, MEI-1 and MEI-2 were co-expressed in 293T cells and their associations were determined by reciprocal immunoprecipitation and western blotting. (**d**) Disruption of Cul3–Roc1 binding enhanced the association of Cul3 and MEI-1. Binding of wild-type and mutant Cul3 with Mel-26 and MEI-1 was determined by immunoprecipitation and western blotting.

lysed with 0.5% NP-40 lysis buffer and lysates were pooled (53 mg total). To purify ectopically expressed Cul3 complexes, two 150-mm plates of 293T cells were transfected with a plasmid expressing Flag-tagged wild-type Cul3 or the $Cul3^{\Delta N41}$ mutant. Cells were lysed with NP-40 lysis buffer and lysates were pooled (estimated 20 mg total). Lysates were immunoprecipitated with affinity purified anti-Cul3 antibody (18 μg) or 30 μl anti-Flag M2–agarose gel (Sigma, St Louis, MO). Immunocomplexes were resolved by SDS–PAGE, stained with Coomassie blue, and the protein bands were digested with trypsin and subjected to mass spectrometric analysis at the UNC Proteomics Core Facility (Chapel Hill, NC).

Plasmids, antibodies and cell culture. Plasmids expressing various wild type and mutant human cullins and ROC1 were described previously[18,30,31]. Full-length human KIAA1309 and KIAA1354 EST clones were obtained from the Kazusa DNA Research Institute (Chiba, Japan). Other human BTB genes were amplified from a HeLa cDNA library. *C. elegans* Cul3, Mei-1, Mei-2 and Mel-26 cDNA were amplified from a *C. elegans* cDNA library (a gift from S. Ahmed, UNC-CH). Procedures for immunoprecipitation and immunoblotting have been described previously[17,31]. A rabbit polyclonal antibody against Cul3 was raised against a synthetic peptide corresponding to the amino-terminal region of human Cul3 (MSNLSKGTGSRK). Antibodies against Roc1 (ref. 30), HA (12CA5; Roche, Indiana, IN), Myc (9E10; NeoMarker, Fremont, CA), T7 (Novagen, Madison, WI) and Flag (M2; Sigma) were previously described or purchased commercially.

Ubiquitin ligase activity assay. The procedures for ubiquitin labelling were as previously described[17,31]. Briefly, to purify substrate, Flag-tagged *C.elegans* MEI-1 was expressed alone or co-expressed with HA-tagged *C. elegans* MEI-2 in 293T cells, immunoprecipitated using affinity-purified Flag antibody and eluted with Flag peptides. To purify Cul3–Roc1 ligase from mammalian cells, appropriate plasmids were transfected into 293T cells. Cul3 immunocomplexes immobilized on protein-A–agarose beads were washed three times with NP-40 cell lysis buffer, and twice with a buffer containing 25 mM Tris-HCl at pH 7.5, 50 mM sodium chloride, 1 mM EDTA, 0.01% NP-40 and 10% glycerol. To purify Cul3–Roc1 from insect cells, Sf9 cells were co-infected with baculoviruses expressing GST–Cul3 and Roc1. Purification of the GST–Cul3–Roc complex was performed in accordance with the manufacturer's instructions (BD Bioscience-Pharmingen, San Diego, CA). Autoubiquitination assays were performed as previously described[17]. For the *in vitro* substrate ubiquitination assay, immunoprecipitated or purified Cul3–Roc was mixed with eluted MEI-1 proteins and mixtures were added to a ubiquitin ligation reaction (final volume 30 μl) containing 50 mM Tris-HCl at pH 7.4, 5 mM $MgCl_2$, 2 mM sodium fluoride, 10 nM okadaic acid, 2 mM ATP, 0.6 mM dithiothreitol (DTT), 12 μg bovine ubiquitin, 1 μg Flag–ubiquitin (Sigma), or 1 μg HA–ubiquitin, 60 ng E1 and 300 ng E2–Ubc5. Reactions were incubated at 37 °C for 60 min, terminated by boiling for 5 min in SDS sample buffer containing 0.1 M DTT, and resolved by SDS–PAGE, before immunoblotting with the anti-Flag antibody to examine ubiquitin ladder formation. Alternatively, reactions were terminated by boiling

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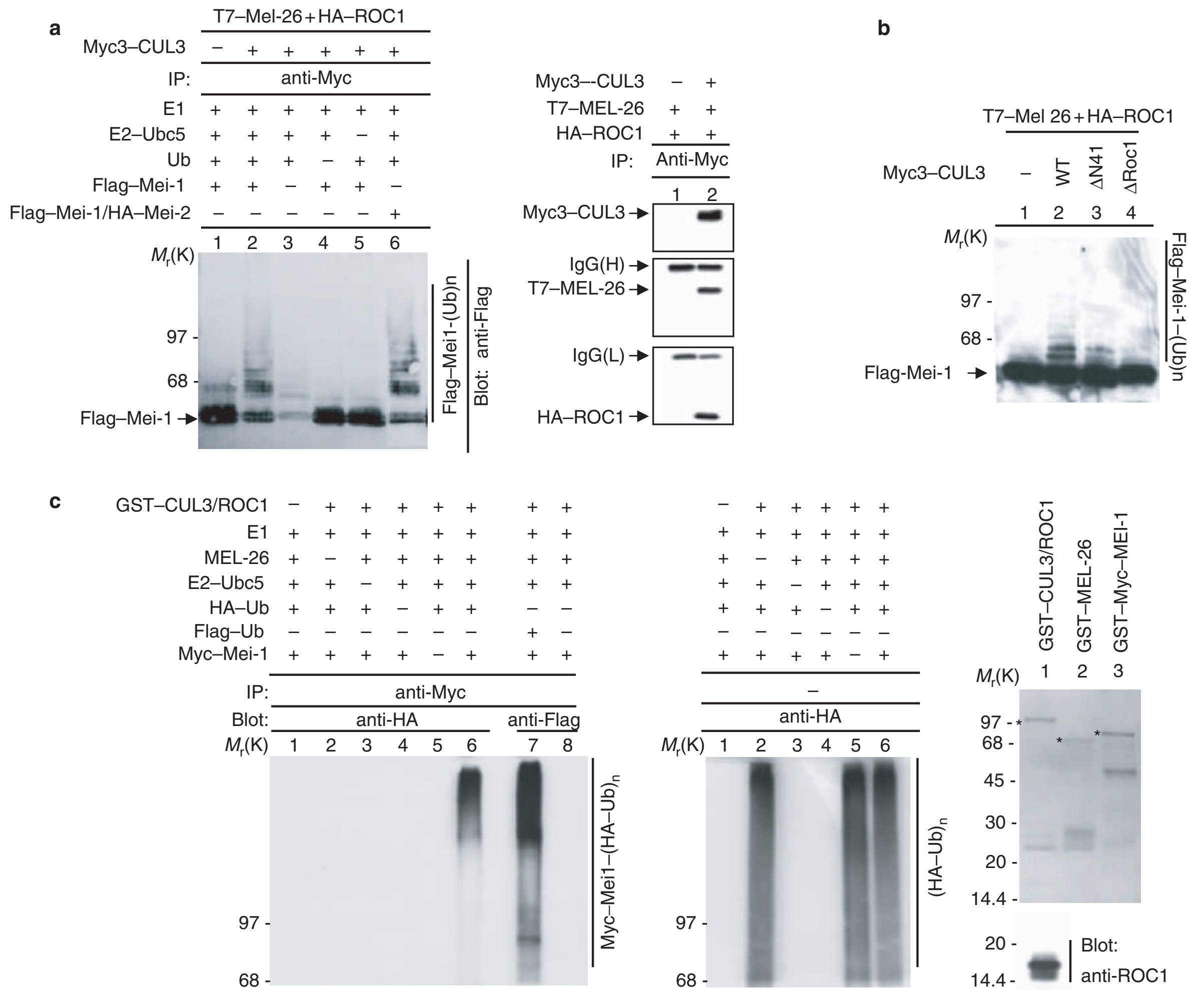

Figure 5 MEL26-Cul3–Roc1 promotes MEI-1 ubiquitination *in vitro*. (**a**) The BTB protein MEL-26, Cul3 and Roc1 were co-expressed in 293T cells, immobilized onto protein A beads and used as the source of E3 ligase. Association of MEL-26 and ROC1 with Cul3 was confirmed by immunoprecipitation and western blotting (right). Flag–MEI-1 or Flag–MEI1–HA–MEI2 was immunoprecipitated, eluted using a Flag peptide and used as a substrate. After incubation with E1, E2 and ubiquitin, reaction mixtures were resolved by SDS–PAGE and analysed by immunoblotting with an anti-Flag antibody. (**b**) *In vitro* ubiquitination of MEI-1 by wild-type and mutant Cul3. *In vitro* ubiquitination of MEI-1 by MEL26-Cul3–Roc1 was performed as described in **a**. (**c**) *In vitro* ubiquitination of MEI-1. GST–Myc–MEI-1 and GST–MEL26 were purified from bacteria and the Cul3–Roc1 complex was purified from insect cells co-infected with baculoviruses expressing both proteins. The purity of each purified component (indicated by an asterisk) was verified by SDS–PAGE and Coomassie staining. Cul3-associated Roc1 was examined by immunoblotting (right). Purified MEI-1 was incubated with Cul3–Roc1 in the presence or absence of various components, as indicated. After *in vitro* ubiquitination, half of the reaction was immunoprecipitated with an anti-Myc antibody, resolved by SDS–PAGE and MEI-1 ubiquitination was examined by two different antibodies recognizing epitope-tagged ubiquitin (left panel). The other half was analysed by SDS–PAGE and immunoblotting with an anti-HA antibody to determine the auto-ubiquitination activity of Cul3–Roc1 (middle).

for 10 min in denaturing buffer containing 50 mM Tris-HCl at pH 7.5, 0.5 mM EDTA, 1% SDS and 1 mM DTT, and diluted with NP-40 cell lysis buffer before immunoprecipitation with the anti-Myc antibody to purify Myc–MEI-1.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.

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