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# Characterization of RhoBTB-dependent Cul3 ubiquitin ligase complexes –Evidence for an autoregulatory mechanism

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## Abstract

RhoBTB proteins are atypical members of the Rho family of small GTPases. Two of the three RhoBTB proteins, RhoBTB1 and RhoBTB2, have been proposed as tumor suppressors and might function as adaptors of Cul3-dependent ubiquitin ligase complexes. Using yeast two-hybrid analysis and co-immunoprecipitation we show that all three RhoBTB proteins interact with Cul3. The interaction requires the N-terminal region of Cul3 and the first BTB domain of RhoBTB. RhoBTB3, the only RhoBTB with a prenylation motif, associates with vesicles that are frequently found in the vicinity of microtubules, suggesting a participation in some aspects of vesicle trafficking. We also show that RhoBTB2 and RhoBTB3 are capable of homo- and heterodimerizing through the BTB domain region. The GTPase domain, which does not bind GTP, is able to interact with the BTB domain region, thus preventing proteasomal degradation of RhoBTB. This fits into a model in which an intramolecular interaction maintains RhoBTB in an inactive state, preventing the formation or the functionality of Cul3-dependent complexes. We also report a significantly decreased expression of RHOBTB and CUL3 genes in kidney and breast tumor samples and a very good correlation in the expression changes between RHOBTB and CUL3 that suggests that these genes are subject to a common inactivation mechanism in tumors.

### Keywords

Rho guanosine triphosphatase; RhoBTB; BTB domain; ubiquitin; cullin; cancer profiling array

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## INTRODUCTION

Rho GTPases are molecular switches primarily implicated in processes that depend on remodeling of the actin cytoskeleton, but they also participate in signaling pathways that regulate gene expression and cell cycle progression [1–3]. Rho GTPases, as well as many of their regulators and effectors, have been implicated in all steps of cancer development [4–6]. Interestingly, with the exception of RhoH and Rac1, no mutations in Rho GTPases have been found associated specifically with tumors. Rather, Rho GTPases have been found overexpressed or hyperactivated in some tumors. For example, overexpression of RhoC in melanoma enhances metastasis, and expression of Rac1b and Cdc42 has been found increased in colorectal cancer and breast tumor samples, respectively [7–9].

The Rho family comprises 21 members (if one includes the highly divergent RhoBTB3) that have been grouped in subfamilies mainly based on sequence, but to some extent also on functional similarities. The RhoBTB subfamily comprises three members whose genes are ubiquitously expressed, although with notable differences in the pattern of tissue levels [10]. Two members, RhoBTB1 and RhoBTB2, have attracted attention as tumor suppressors [11]. RHOBTB2 (also known as DBC2, deleted in breast cancer 2) was identified as the gene homozygously deleted at region 8p21 in breast cancer samples and was proposed as a candidate tumor suppresor gene based on the fact that its re-expression in breast cancer cells lacking RHOBTB2 transcripts caused growth inhibition, whereas expression of a somatic mutant previously identified in a breast cancer specimen did not have the same effect [12]. In subsequent studies high rates of loss of heterozygosisty at the RHOBTB2 locus have been found in gastric tumors as well as in bladder tumors and cell lines [13,14]. Over the years several somatic missense mutations have been identified in the coding region and in the promoter and 5’UTR of the RHOBTB2 gene [12–15]. In a recent study RHOBTB1 has also been postulated as a tumor suppressor gene in head and neck squamous cell carcinomas, although no pathogenic mutation was found [16].

Rho GTPases of the RhoBTB subfamily have an unusual domain architecture [10,17]. The GTPase domain is followed by a proline rich region, a tandem of two BTB (Broad complex, Tramtrack, Bric à brac) domains and a conserved C-terminal region. The GTPase domain is apparently non-functional [18] and in RhoBTB3 it is almost no longer recognizable as such. Only RhoBTB3 ends with a CAAX box, a motif characteristic of most Rho GTPases. The CAAX box is a signal for isoprenylation, a post-translational modification that enables association to membranes. The BTB domain is an evolutionary conserved domain found widespread among eukaryotes. It has been known for long time as a protein-protein interaction domain participating in homomeric and heteromeric associations with other BTB domains. Recently BTB domains have been shown to function as adaptors in cullin 3-dependent ubiquitin ligase complexes [19–22].

Cullins function as scaffolding proteins that bring together the ubiquitin conjugating enzyme and substrate recognition components for ubiquitin mediated degradation by the 26S proteasome [23]. Substrate selection is determined by the binding of adaptors to the N-terminal region of the cullin, for example Skp1 and substrate-specific F-Box protein in Cul1 complexes and ElonginC and BC-Box-containing proteins in Cul2/5 complexes. Skp1, ElonginC and BTB contain a conserved fold (the so called BTB core) despite a low degree of primary sequence conservation [24]. The BTB domains of RhoBTB, like those of proteins of the BTB-ZF, BTB-Kelch and MATH-BTB families, contain each an N-terminal extension responsible for the homo and heteromeric association reported for several BTB proteins [24]. Cul3 was found as a binding partner of RhoBTB2 and is itself a substrate for Cul3-dependent ubiquitinylation [25], suggesting the possibility that BTB proteins can act as substrate adaptors and/or direct substrates of the Cul3-dependent ligase.

The roles of RhoBTB proteins in general, and in cancer in particular, remain largely unexplored, but an effect on the organization of the actin cytoskeleton has been ruled out [11,26]. The evidence gathered so far is very restricted and points at roles in the regulation of cell growth and vesicle trafficking, but the mechanisms are virtually unknown [18,27–29]. Here we have explored the requirements for the formation of RhoBTB-dependent Cul3 complexes We found that all three RhoBTB proteins interact specifically with Cul3 and that this interaction requires the first BTB domain of RhoBTB. We also found that the BTB domains are involved in the formation of homo and heterodimers and that the GTPase domain interacts with the BTB domains. We propose a model in which an intramolecular interaction between the GTPase domain and the BTB domains prevents interaction with Cul3 and possibly maintains RhoBTB in an inactive state. This model is underscored by a significantly decreased expression of RHOBTB and CUL3 genes in kidney and breast tumor samples and a very good correlation in the expression changes between RHOBTB and CUL3 that suggests that they may be co-regulated.

## MATERIALS AND METHODS

### Plasmids

A list of all constructs used in this study is shown in Suppl. Table 1. Myc-tagged cullins [19] were subcloned into an appropriate yeast vector. Dominant negative Flag-tagged cullin constructs are a generous gift from Wade Harper, Harvard Medical School, Boston, USA. Human and mouse RhoBTB1, 2 and 3 [10,26] and fragments thereof (Figure 1A) were subcloned into appropriate vectors for expression as GFP, Myc tag or Flag tag fusions in mammalian cells, for expression in *E. coli* as GST fusions or for expression in yeast using standard techniques.

### Cell culture and transient transfection

COS7, HeLa, 293T and PAE/PDGFRβ (stably expressing the human PDGF β-receptor) cells were cultivated using standard procedures. For immunofluorescence all cell lines were seeded on coverslips, transfected using Lipofectamine (Invitrogen, Karlsruhe, Germany) according to the protocol provided by the manufacturer and cultivated for 24 h unless otherwise indicated. For immunoprecipitation studies COS7 cells were grown on 10 cm plates, transfected with a DEAE-PBS-DNA solution for 30 min, incubated with 100 μM chloroquine in DMEM for three hours and then returned to DMEM for 40 h. For immunoprecipitation 293T cells were transfected using Lipofectamine. Where indicated, cells were treated with with 100 μM cycloheximide, 5–25 μM proteasomal inhibitor MG132, (Sigma, Taufkirchen, Germany) or with DMSO as a control.

### Immunoprecipitation

Immunoprecipitation was done in two ways. Transfected cells were lysed with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100 and protease inhibitors for 30 minutes. After clearing by centrifugation at 10,000×*g* at 4°C, immunoprecipitation was performed using a μMACS epitope tag protein isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Alternatively, cells were lysed with 20 mM Hepes pH 7.5, 1% Triton X-100, 10% glycerol, 100 mM NaCl and 5 mM EDTA for 10 min. After clearing by centrifugation, the supernatant was mixed with agarose-conjugated anti-Myc antibodies (Santa Cruz, Heidelberg, Germanyfor 2 h. After four washing steps with lysis buffer, elution was done with 10 μl sample buffer. Samples were analyzed by SDS-PAGE and western blot. Western blots were quantitated using AlphaEase software (Alpha Innotech, San Leandro, CA)

### Immunofluorescence

Cells were fixed in methanol (10 min at −20°C) or 3% paraformaldehyde in PBS (20 minutes at 37°C) and washed with PBS. In both cases cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed again in PBS and incubated in 5% FBS in PBS for 30 min at room temperature. . Primary as well as secondary antibodies were dilutetd in PBS containing 1% FBS an applied for intervals of 1 h with a washing step in between. Filamentous actin was visualized with TRITC-labeled (Sigma) or Alexa Fluor 350-labeled (Molecular Probes, Karlsruhe, Germany) phalloidin. Nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) (Sigma, Taufkirchen, Germany). The coverslips were mounted on object slides using gelvatol as embedding medium. Conventional fluorescence images were taken in a Zeiss Axioplan2 microscope equipped with a Hamamatsu ORCA CCD digital camera. Confocal images were taken with an inverted Leica TCS-SP laser-scanning microscope with a 100x HCX PL APO NA 1.40 oil immersion objective. For excitation, the 488 nm argon-ion laser line and the 543 nm HeNe laser line were used.

### Ubiquitinylation and protein stability assays

The in vivo ubiquitinylation assay was carried out as previously described [19]. Briefly, 293T cells were transfected with appropriate plasmids. 20 hours after transfection cells were treated with 25 μM MG132 for 4 h prior to cell lysis. Cells were lysed in a 1% SDS-containing buffer and boiled for 15 min. Lysates were then diluted to 0.1% SDS and immunoprecipitated with anti-Myc antibody. Washed immunoprecipitates were resolved by SDS-PAGE and immunobloted for detection of the polyubiquitinylated protein.

To study protein stability 293T cells were transfected with appropriate plasmids. 16 hours after transfection cells were split and treated with 100 μM cycloheximide and either 10μM MG132 or DMSO as a control, as described [25]. At indicated time points cells were lysed and analysed for presence of tagged proteins.

### Antibodies

Following primary antibodies were used: anti-GFP (mAb K3-184-2, [30]), anti-Myc (mAb 9E10 and rabbit polyclonal; Epitomics, Burlingsame, USA or Santa Cruz), anti-Flag (Acris, Hiddenhausen or Sigma, Taufkirchen, Germany), anti-hemagglutinine (HA) (Covance, Princeton, NJ), anti β-actin (mAb AC-74, Sigma), anti-PDI (Stressgen Biotechnologies, Victoria, Canada), anti-α-tubulin (Calbiochem, San Diego, CA, USA) and anti-EEA1 (Transduction Laboratories, Heidelberg, Germany). A rabbit polyclonal anti-Cul3 antibody recognizing the C-terminus of human Cul3 was generated against following peptide: QGESDPERKETRQKVDDDRKHEIE. As secondary antibodies we used for immunofluorescence Alexa Fluor 568 or 488 conjugated anti-mouse and anti-rabbit immunoglobulins (Molecular Probes) and TRITC-conjugated anti-mouse immunoglobulin (Jackson ImmunoResearch, West Groove, PA, USA) and for western blot peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin (Sigma).

### Yeast two-hybrid analysis

The protocols of the Matchmaker Two-hybrid system from Clontech (BD Biosciences Clontech, Palo Alto, CA, USA) were followed for all experiments dealing with two-hybrid assays. Bait and prey DNA fragments were cloned into pACT/pAS and pGADT7/pGBKT7 vectors (Suppl. Table 1). *S. cerevisiae* strains Y187 and Y190 were cultivated using standard methods. Interactions were assessed semi-quantitatively by colony-lift β-galactosidase filter assay on colonies grown on plates lacking tryptophan and leucine after mating. All constructs were tested for autoactivation.

### Human cancer expression arrays

A cancer profiling array was obtained from Clontech. It contains SMART™ cDNA synthesized from Clontech's premium RNA™ from 241 cases of matched normal and tumor tissue from individual patients. The array was hybridized with RHOBTB and CUL3 probes as indicated in Suppl. Figure 4. Radiolabeled human ubiquitin cDNA was used to ensure the integrity of the samples on the array and to normalize the signals for quantification. The array was analyzed using a Phosphorimager 445SI (Molecular Dynamics, Krefeld, Germany) and ImageQuant Software. Statistical analysis was performed using Excel.

### Miscellaneous methods

Standard molecular biology methods were used as described [31]. All PCR products and all constructs were verified by sequencing, done at the service laboratory of the Center for Molecular Medicine, Cologne, using an automated sequencer (ABI 377 PRISM, Perkin Elmer, Norwalk, CO).

## RESULTS

### RhoBTB proteins interact with Cul3

It has been described that RhoBTB2 interacts physically with Cul3 [25]. We first investigated whether all three RhoBTB proteins, in particular RhoBTB3, had the same Cul3 binding ability. We found that all three ectopically expressed RhoBTB proteins were able to co-immunoprecipitate endogenous Cul3 (Figure 1B). The reciprocal experiment was not possible, as a polyclonal antibody raised against RhoBTB3 was not able to detect any endogenous protein. In order to map the interaction more precisely, we performed immunoprecipitation experiments with deletion mutants of RhoBTB3 consisting of the individual BTB domains or the C-terminal extension. Single domains of RhoBTB3 were expressed as GFP-fusion in COS7 cells together with Myc-tagged Cul3. Complexes were immunoprecipitated and analyzed for the presence of GFP fusion proteins. Only those fusion proteins encompassing the first BTB domain co-immunoprecipitated with Cul3. The first BTB domain of RhoBTB3 is therefore necessary and sufficient for interaction with Cul3 (Figure 1C). It appears that both BTB domains together result in a stronger interaction with Cul3 than the first BTB domain alone.

We used a yeast two-hybrid approach to determine whether Cul3 is the only cullin able to interact with RhoBTB3 (not shown). With this approach we observed an interaction of the C-terminal (B1B2C) region of RhoBTB3 with Cul3 and, surprisingly, also Cul5, whereas the GTPase domain did not interact with any cullin. As expected RhoBTB2 interacted with Cul3, but also with Cul5. A deletion mutant lacking the first 41 residues (Cul3ΔN41) was used to verify that the interaction requires the N-terminus of Cul3 [19]. Similarly, using N and C-terminal deletion mutants we observed that the interaction requires the first 93 residues of Cul5 needed to bind ElonginC [32], but not the last 200 residues.

To confirm the interactions observed with the yeast two-hybrid approach, we transfected COS7 cells with Flag-tagged RhoBTB3 and Myc-tagged cullins. RhoBTB3 was immunoprecipitated and the immune complexes analyzed by Western blotting for the presence of cullin. These experiments yielded inconsistent and irreproducible results that suggested that RhoBTB3 is able to interact with virtually all cullins. Similar results were obtained when RhoBTB1 or RhoBTB2 were used (not shown). Moreover, in immunofluorescence experiments a co-localization of RhoBTB3 with all cullins, including Cul3ΔN41, was apparent (Suppl. Figure 1).

We suspected that RhoBTB proteins directly interact with one cullin, but are then able to form multimolecular complexes that incorporate other cullins, presumably either through

components that interact with the C-terminus of cullin or through cullin heterodimerization. We therefore repeated the immunoprecipitation approach using Flag-tagged dominant negative (DN) cullins and Myc-tagged RhoBTB. DN cullins lack the C-terminal region needed for interaction with the RING-box protein, and therefore do not display catalytic activity [23]. All three RhoBTB isoforms immunoprecipitated DN-Cul3 only. DN-Cul5 was not found in these immune complexes (Figure 2A). The reciprocal immunoprecipitation experiment using Flag-tagged RhoBTB and Myc-tagged DN-cullins yielded a comparable result (not shown). In immunofluorescence experiments a co-localization of RhoBTB with DN-Cul3, but not DN Cul5, was apparent (Figure 2B–D).

## RhoBTB proteins form homo and heterodimers

The BTB domain is considered a protein-protein interaction module able to undergo interactions with itself and with proteins without BTB domains [24]. We used a yeast two-hybrid approach to investigate whether RhoBTB proteins are also able to dimerize (not shown). We observed an interaction of RhoBTB3-B1B2C with itself. The extension C-terminally to the BTB domains was not required for the interaction, although it may have a stabilizing effect, because the interaction appeared stronger when it was present. A homodimerization of RhoBTB2 was also observed, as reported by others [33]. In addition we found that RhoBTB2 interacts with RhoBTB3-B1B2C, indicating that RhoBTB proteins are also capable of heterodimerizing. In this experiments we used full-length RhoBTB2, therefore we cannot exclude that the GTPase domain mediates the interaction. We therefore assayed the GTPase domain of RhoBTB3 and RhoBTB2 for interaction with RhoBTB3-B1B2C and observed a strong interaction of both GTPase domains that we nailed down to the BTB tandem. No interaction of other Rho GTPases (Cdc42, RhoA, Rac1) to RhoBTB3-B1B2C was observed, irrespective of the activation state of the GTPase. We have incidentally noticed that the GTPase domain of RhoBTB3 does not bind GTP (Suppl. Figure 2), a property also reported for RhoBTB2 [18].

The interactions observed with the yeast two-hybrid approach were verified in vivo in co-immunoprecipitation experiments. Both RhoBTB2 and RhoBTB3 were able to co-immunoprecipitate RhoBTB3 as well as the GTPase domain of RhoBTB3 (Figure 3A). RhoBTB3 was also able to co-immunoprecipitate the GTPase domain of RhoBTB2. The ability of RhoBTB proteins to form heterodimers was supported by colocalization of GFP-tagged RhoBTB3 and Flag-tagged RhoBTB2 in COS7 cells (Figure 3B).

In summary, these results indicate that RhoBTB proteins exist as homo and/or heterodimers, that dimerization occurs through the BTB domains and that the GTPase domain is also able to interact with the BTB domains. To map more precisely the region involved in dimerization, we transfected COS7 cells with Myc-tagged RhoBTB2-B1B2C and GFP-tagged single BTB domains of RhoBTB3. After immunoprecipitation with anti-Myc antibodies, both the first and the second BTB domain were detected in the immune complexes, indicating that both BTB domains are involved in the formation of dimers (Figure 3C).

## RhoBTB3 associates with vesicles

When transfected into mammalian cells, all three RhoBTB proteins accumulated in paranuclear aggregates in all cells lines studied (Figure 2D) [26]. However, when expressed at moderate levels, RhoBTB3 displayed a vesicular pattern. These vesicles were of comparable sizes and accumulated predominantly in the paranuclear region, surrounding the MTOC, with isolated vesicles dispersed throughout the cytoplasm (Figure 4). Counterstaining for tubulin revealed that GFP-RhoBTB3-labeled vesicles frequently appeared associated with microtubules. Counterstaining for actin revealed some instances of close proximity of the vesicles to stress fibers in peripheral regions (Figure 4, arrows).

In order to identify the nature of the vesicles we stained GFP-RhoBTB3 expressing cells with antibodies specific for the early endosome marker EEA1 and observed some instances of co-localization. Very restricted targeting to membranes of the endoplasmic reticulum was observed upon co-staining for protein disulfide isomerase (Figure 4). To assess which part of RhoBTB3 determines its subcellular localization, we generated diverse deletion constructs of this protein for expression as GFP fusions and observed that the C-terminal extension is necessary and sufficient for attaching of RhoBTB3 at vesicles (Suppl. Figure 3). This property is apparently conferred by isoprenylation (RhoBTB3 is the only RhoBTB protein that harbors a prenylation motif), as a C-terminal fragment in which the isoprenylation motif was deleted displayed a diffuse localization all over the cell.

## RhoBTB3 is degraded in the 26S proteasome

RhoBTB2 is a substrate of Cul3-dependent ubiquitin ligase complexes [25]. Because RhoBTB3 also interacts with Cul3, we investigated the levels of GFP-tagged RhoBTB3 after proteasome inhibition. After 24 h of treatment with MG132, the cells showed a clear accumulation of the fusion protein when compared with cells treated with the vehicle. This difference was apparent both by visual inspection (Figure 5A) and in western blot (Figure 5B). In an in vivo assay we observed accumulation of polyubiquitinylated RhoBTB3 upon treatment with MG132. The amount of polyubiquitinylated RhoBTB3 decreased in the presence of Cul3 ΔROC1, a dominant negative mutant Cul3 that binds to BTB proteins and substrates but cannot promote ubiquitinylation [19] (Figure 5C).

The interaction of the GTPase domain with the BTB tandem suggests a model in which the GTPase domain prevents either interaction of the first BTB domain with Cul3 or subsequent ubiquitinylation. We therefore reasoned that in the presence of the GTPase a C-terminal fragment encompassing the BTB domain would be more stably expressed. To test this hypothesis we performed a protein stability experiment. We transfected 293T cells with GFP-tagged RhoBTB3-B1B2C or both the Myc-tagged GTPase domain and the GFP-tagged B1B2C region of RhoBTB3 and arrested protein biosynthesis. Lysates were analyzed for the amounts of Myc and GFP-tagged protein. As expected, RhoBTB3-B1B2C accumulated at higher levels when co-expressed with the GTPase domain (Figure 5D, left hand panels), in spite of the GTPase degrading rapidly. In an analogous experiment full length RhoBTB3 behaved more stably, and the presence of the GTPase had no noticeable effect on its stability (Fig. 5D, right hand panels).

## Reduced expression of RHOBTB and CUL3 in tumor tissues

RhoBTB proteins and Cul3 being part of the same complex, we suspected that expression of the corresponding genes could be altered in similar ways in tumor tissues. A cancer profiling array was hybridized with radiolabeled RHOBTB1, RHOBTB2, RHOBTB3 and CUL3 probes to determine changes in their pattern of expression between normal and tumoral tissue (Suppl. Figure 4). Hybridization with a RHOBTB2 probe did not yield signals significantly above the background, therefore this gene was not considered for further analyses. In fact, RHOBTB2 is very weakly expressed in tissues compared to RHOBTB1 and RHOBTB3 [10,34]. Several tumor tissues presented decreased expression of the genes studied when compared to the matched normal tissue (Figure 6). For RHOBTB1 the average decrease was statistically significant in kidney (~1.9 fold, n = 20) and breast (~1.4 fold, n = 50) followed by stomach (~1.5 fold, n = 27). RHOBTB3 expression was significantly decreased in kidney (~2.3 fold), breast (~1.7 fold) and uterus (~1.4 fold, n = 42), followed by lung (~1.45 fold, n = 21) and ovary (~1.9 fold, n = 14). Expression of CUL3 was significantly decreased in breast (~1.5 fold) and kidney (~1.8 fold).

In 50 cases of breast tissue, 29 cases (58%) of RHOBTB1, 28 cases (56%) of RHOBTB3 and 37 cases (74%) of CUL3 demonstrated decreased expression of 1.4-fold or more in the tumor tissue versus the matched normal tissue. Considering the same 1.4-fold cutoff, from 20 cases of kidney tissue, 16 cases (80%) of RHOBTB1, RHOBTB3 and CUL3 showed a decreased expression in tumoral as compared to normal tissue. All three genes were simultaneously down regulated above the 1.4-fold cutoff in 15 cases (30%) of breast and in 11 cases (55%) of kidney tumor and no cases of simultaneous up regulation were found. In general, expression of all three genes did not differ in metastatic samples compared to the corresponding tumor sample.

### Expression of RHOBTB and CUL3 is correlated

The observation that expression of RHOBTB1, RHOBTB3 and CUL3 is decreased simultaneously in some tissues led us to analyze in more detail whether there is a correlation in the expression levels of these genes (Table 1). We observed significant correlation between RHOBTB3 and CUL3 ($r = 0.3390$, $P = 8.7 \times 10^{-7}$) and between RHOBTB1 and RHOBTB3 ($r = 0.2892$, $P = 6.7 \times 10^{-6}$), and more weakly between RHOBTB1 and CUL3 ($r = 0.1526$, $P = 0.0187$). Focused on single tissues, the correlation between RHOBTB3 and CUL3 was high in breast and uterus, followed by lung and rectum, and the correlation between RHOBTB1 and CUL3 was high in breast, uterus and stomach.

## DISCUSSION

Here we show that the ability to form Cul3-dependent complexes is shared by all three RhoBTB proteins. As expected, the interaction requires the N-terminus of Cul3 [19]. Although, intriguingly, RhoBTB may potentially also interact with the N-terminus of Cul5, as observed in the yeast two-hybrid approach, this interaction does not seem to be favored when RhoBTB and Cul5 meet in mammalian cells. The first BTB domain of RhoBTB3 is involved in binding to Cul3, as already described for RhoBTB2 [25]. In vivo Cul3-dependent complexes may incorporate other cullins, and in fact recent evidence suggest that cullins function as homo and heterodimers in vivo [35,36]. This would explain the need to remove the C-terminal region of the cullin in order to observe specific interaction of Myc-tagged RhoBTB with Cul3 in immunoprecipitation and colocalization studies. Wilkins et al. [25] specifically immunoprecipitated full length Cul3 with GST-tagged RhoBTB. It is very likely that the bulky GST tag hindered the formation of larger complexes.

RhoBTB proteins apparently exist as homo- and heterodimers, and both BTB domains participate in dimerization. There are many examples of proteins that dimerize through the BTB domain (see [24] and references therein), and several are recognized Cul3 adaptor proteins, like the promyelocytic leukemia zinc finger (PLZF) protein [19] and Keap1 [37]. In fact, dimerization has been proposed as a general feature of Cul3 substrate adaptors [33]. The BTB domains of RhoBTB proteins contain each an N-terminal extension that in other BTB domains folds into one α-helix and one β-strand and mediates the formation of oligomers [24]. It remains to be established whether the dimer is parallel or antiparallel (meaning in this case that the first BTB domain of one monomer interacts with the second BTB domain of the other monomer), but the ability to form heterodimers would rule out the formation of an intramolecular dimer, as proposed by Stogios et al. [24]. We show that the GTPase domain, which is able to bind to the C-terminal region of the protein that comprises the BTB tandem, could be responsible for an intramolecular interaction that either blocks the formation of a Cul3-dependent complex or otherwise hinders its ubiquitinylation activity. Unlike in most Rho GTPases, in RhoBTB proteins the GTPase domain does not function as a switch; it even appears not to bind GTP ([18] and this paper).

We propose a model (Figure 7) in which interaction of the GTPase domain with unknown proteins would relieve the autoinhibitory mechanism., The GTPase and other domains, as well

as the insertion of the first BTB domain could function as substrate recognition domains. In this process RhoBTB proteins become ubiquitinylated and degraded. The exact roles of RhoBTB-dependent complexes and how these roles relate to tumor formation are unknown. The localization of RhoBTB3 suggests that this protein participates in vesicle transport, a role already proposed for RhoBTB2. Knockdown of endogenous RhoBTB2 hindered the ER to Golgi apparatus transport and resulted in altered distribution of the vesicular stomatitis virus glycoprotein [18]. In this study the authors found GFP-RhoBTB2 distributed in a vesicular pattern when expressed at low levels. Some of the vesicles appeared adjacent to microtubules and an intact microtubule network seemed required for the mobility of RhoBTB.

Our analysis of the expression profile of RHOBTB and CUL3 genes revealed a significant average decrease of expression of RHOBTB1, RHOBTB3 and CUL3 simultaneously in breast and kidney tumors and of RHOBTB1 and RHOBTB3 alone in some additional tissues. In general the degree of downregulation was on average moderate (not far above 2-fold decrease) but was found in a very large proportion of samples within a tissue group. Remarkably, downregulation of RHOBTB and CUL3 appears not to be an alteration associated with cancer in general, but with particular tumor types, notably breast and kidney. This is also in agreement with the data on RHOBTB2 for bladder, breast and lung cancers [12,13] and on RHOBTB1 in squamous cell carcinomas [16]. In addition, in some tumor types apparently only one of the genes studied was downregulated, such as RHOBTB3 in uterus, ovary and lung tumors. Because RHOBTB2 is infrequently found mutated in tumors but downregulation is frequent, it has been proposed that alternative mechanisms of inactivation are more common [13]. In support of an epigenetic regulatory mechanism, the promoter region of all three RHOBTB genes shows a high GC content with CpG islands, and promoter hypermethylation of RHOBTB2 has been reported recently in bladder cancer [38]. We anticipate that RHOBTB1 and RHOBTB3 will also be found infrequently mutated in tumors. The good correlation in the expression changes between RHOBTB and CUL3 suggests that these genes underlay a common inactivation mechanism in tumors that is worth elucidating in future. Cul3 is widely expressed in mammalian tissues [39,40] and inactivation of the CUL3 locus results in early embryonic lethality in mouse [40], indicating that this cullin plays an essential regulatory role. Considering that there are about 200 genes encoding BTB proteins in the human genome, Cul3-dependent complexes might control ubiquitinylation and degradation of cancer-related proteins through multiple mechanisms. In fact, several BTB proteins have been found linked to tumorigenesis, although their roles in the formation of Cul3-dependent complexes have generally not been addressed [42].

In summary, in analogy to the well established Cul2-pVHL model of tumorigenesis [43], we favor a model in which RhoBTB proteins target specific substrates for ubiquitinylation and degradation via Cul3-dependent ubiquitin ligase complexes. Suppression or downregulation of RHOBTB and CUL3 genes, as seems to occur in particular tumors, would result in accumulation of the cancer targets and cell proliferation. Identifying these targets is now imperative to uncover the signaling pathways in which RhoBTB proteins are involved.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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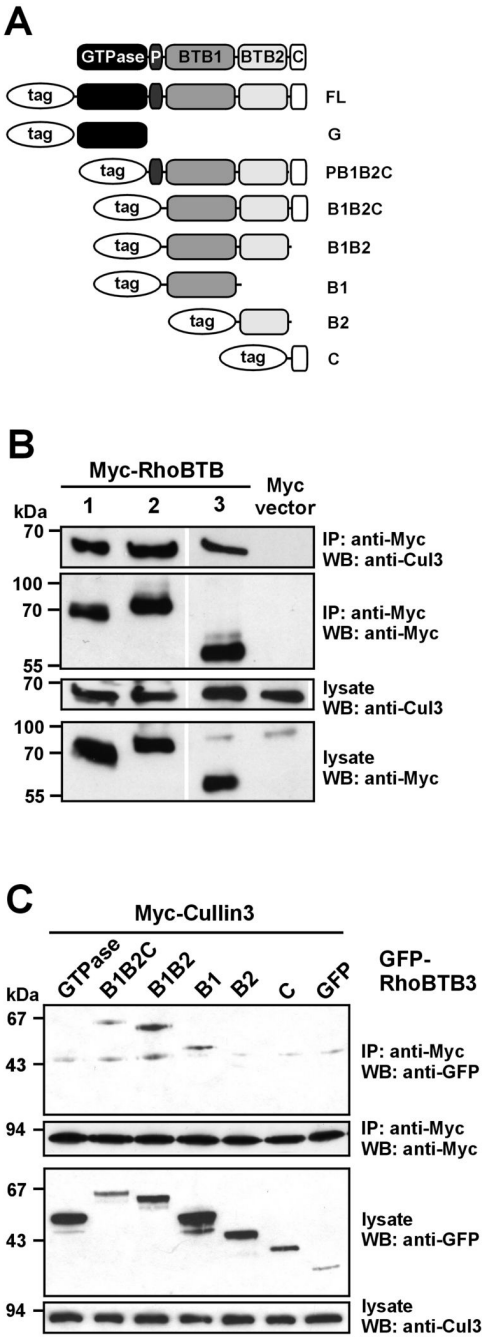


**Figure 1. RhoBTB proteins interact with Cul3 through their first BTB domain**

(**A**) Domain architecture of RhoBTB proteins and constructs used in this study. RhoBTB proteins consist of a GTPase domain followed by a proline-rich region (P), a tandem of two BTB domains and a conserved C-terminal region (C). Full length proteins or the fragments indicated in the figure were expressed as fusions with a tag as indicated throughout the paper and summarized in Suppl. Table 1. The tags used are GFP, Myc epitope, Flag epitope, GST and the DNA-binding or the activation domain of the yeast two-hybrid vectors. (**B**) All three RhoBTB proteins interact with endogenous Cul3 in vivo. 293T cells were transfected with Myc-tagged RhoBTB proteins or the empty Myc vector and treated with proteasomal inhibitor MG132 (5 μM) for 24 hours. After lysis complexes were immunoprecipitated as in B and

analyzed for the presence of endogenous Cul3 and the corresponding Myc-tagged RhoBTB by Western blotting. Lysates were analyzed for the presence of the corresponding input proteins. (**C**) Mapping of the domain of RhoBTB needed for interaction with Cul3. COS7 cells were transfected with single domains of RhoBTB3 as GFP-fusion and Myc-tagged Cul3. Complexes were immunoprecipitated as in B and analyzed for the presence of Myc and GFP fusion proteins by Western blotting. Lysates were analyzed for the presence of the corresponding input proteins. The first BTB domain of RhoBTB3 is necessary and sufficient for interaction with Cul3

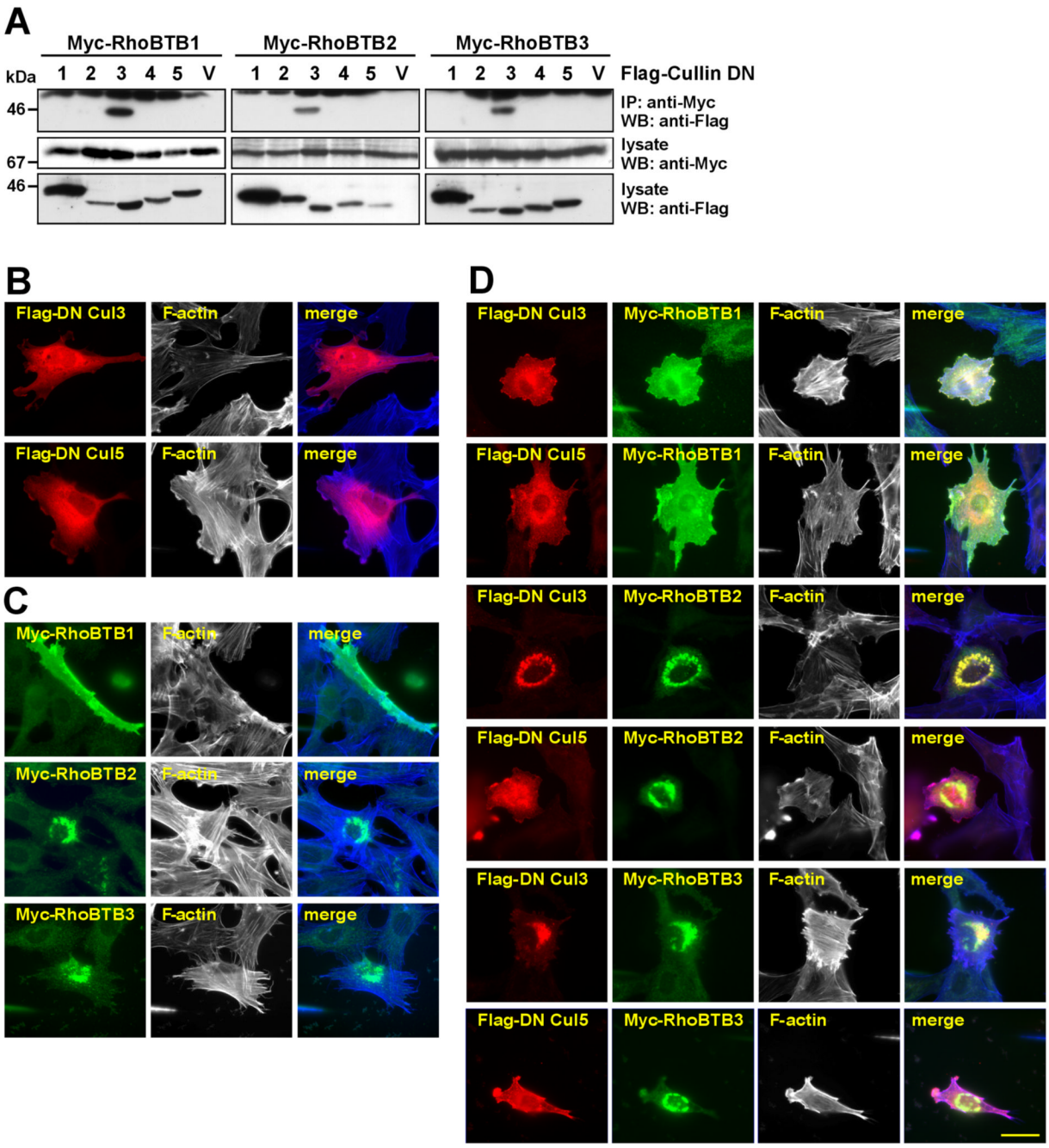


**Figure 2. RhoBTB proteins interact with Cul3 *in vivo.***

(**A**). COS7 cells were co-transfected with Myc-tagged RhoBTB proteins and Flag-tagged dominant negative (DN) cullins. DN cullins lack the C-terminal region needed for interaction with Roc1, also known as Rbx1. V denotes the empty Myc vector. Complexes were immunoprecipitated with anti-Myc antibodies coupled to magnetic beads and analyzed for the presence of cullins by Western blotting. Lysates were analyzed for the presence of the corresponding input proteins. All three RhoBTB proteins interact only with Cul3. (**B–D**) Colocalization of RhoBTB proteins with dominant negative cullins. PAE cells were transfected with the indicated Flag-tagged DN Cul3 or Cul5 and Myc-tagged RhoBTB1, RhoBTB2 or RhoBTB3. Cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton

X-100 and immunostained with mouse anti-Flag and rabbit anti-Myc antibodies followed by TRITC-coupled anti-mouse and Alexa Fluor 568-coupled anti-rabbit secondary antibodies. F-actin was visualized with Alexa Fluor 350-phalloidin. Images were taken with a fluorescence microscope. (**B**) Cells transfected with DN Cul3 or Cul5 only. (**C**) Cells transfected with the individual RhoBTB vectors. (**D**) Cells co-transfected with the indicated DN cullin and RhoBTB. RhoBTB proteins show colocalization with DN Cul3. Bar represents 50 μm.

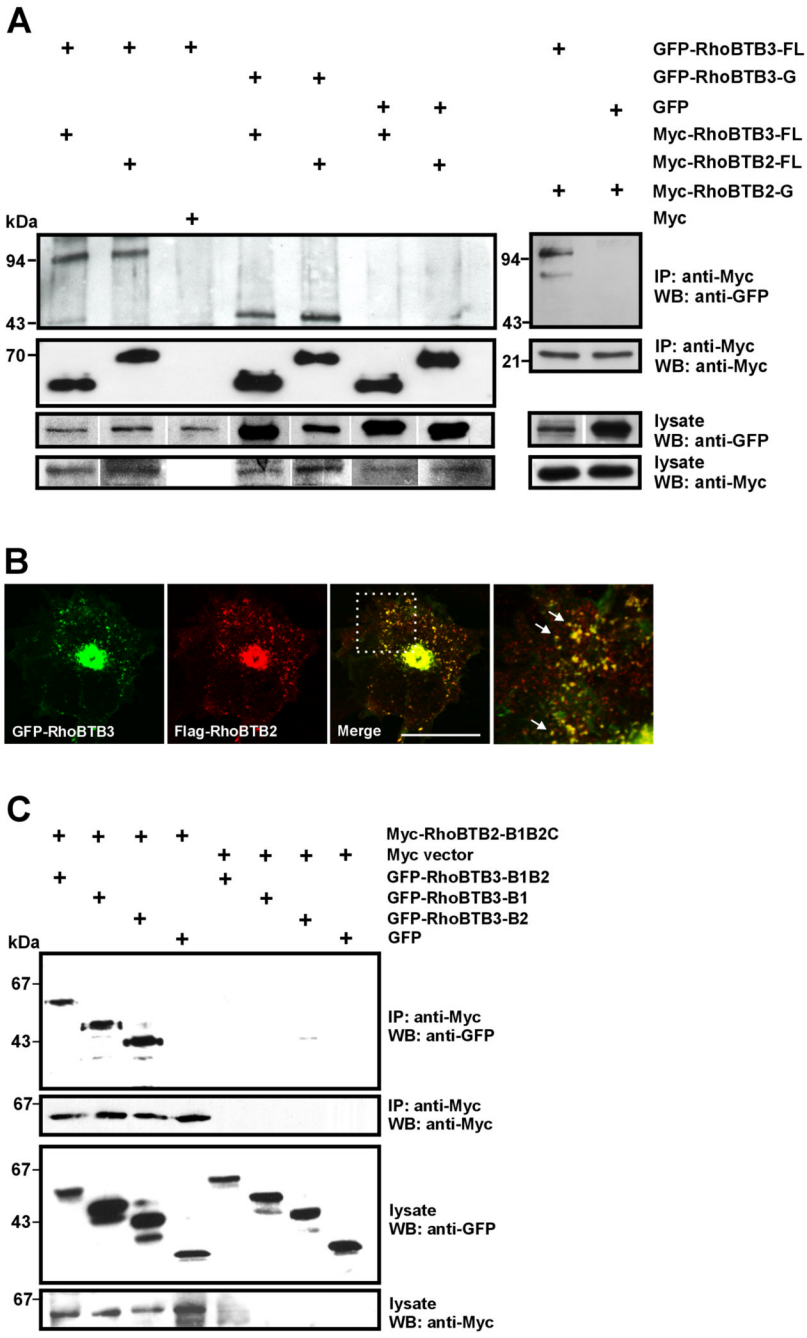


**Figure 3. RhoBTB proteins form homo and heterodimers *in vitro* and *in vivo***

(**A**) COS7 cells were co-transfected with the indicated GFP and Myc-tagged RhoBTB proteins. See Fig. 1A for nomenclature of the constructs. Complexes were immunoprecipitated with anti-Myc antibodies coupled to magnetic beads and analyzed for the presence of Myc and GFP fusions by Western blotting. Lysates were analyzed for the presence of the corresponding input proteins; here the bands have been cut and arranged in a row The reciprocal immunoprecipitations using anti-GFP antibodies yielded comparable results. (**B**) Co-localization of RhoBTB2 and RhoBTB3. COS7 cells were transfected with GFP-tagged RhoBTB3 and Flag-tagged RhoBTB2. After fixation with 3% paraformaldehyde cells were permeabilized with 0.5% Triton X-100 and immunostained. Images were taken with a confocal

laser scanning microscope. An experiment in which the tags were swapped yielded the same result. The magnified region of the rightmost panel corresponds to the square in the merge panel. Arrows highlight instances of colocalization. Bar represents 25 μm. **(C)** Mapping of RhoBTB heterodimerization. COS7 cells were co-transfected with the Myc-tagged B1B2C region of RhoBTB2 and the indicated GFP-tagged domains of RhoBTB3. See Fig. 1A for nomenclature of the constructs. Complexes were immunoprecipitated with anti-Myc antibodies coupled to magnetic beads and analyzed for the presence of GFP fusion proteins. Lysates were analyzed for the presence of the corresponding input proteins. Both BTB domains of RhoBTB3 appear to interact with the B1B2C region of RhoBTB2 *in vivo*.

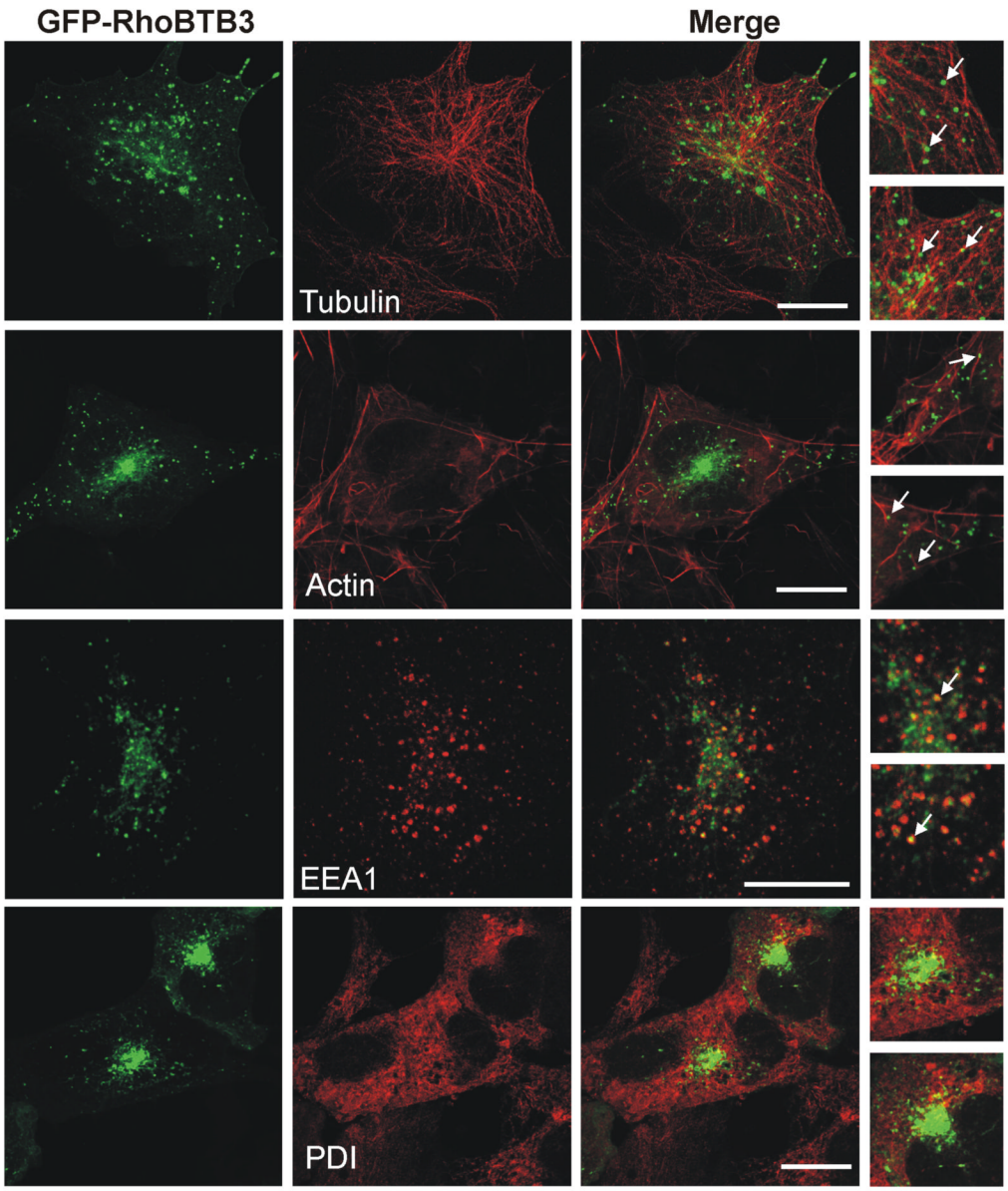


**Figure 4. Subcellular localization of RhoBTB3**

COS7 cells were transfected with GFP-tagged RhoBTB3 constructs, fixed with methanol (for tubulin staining) or 3% paraformaldehyde and stained for different subcellular structures: anti-tubulin antibodies, anti-EEA1 (early endosome antigen 1) antibodies, TRITC-labeled phalloidin and anti-PDI (endoplasmic reticulum) antibodies. Images were acquired with a confocal laser-scanning microscope. Bars represent 25 μm. Arrows in the magnified sections indicate instances of colocalization or close proximity.

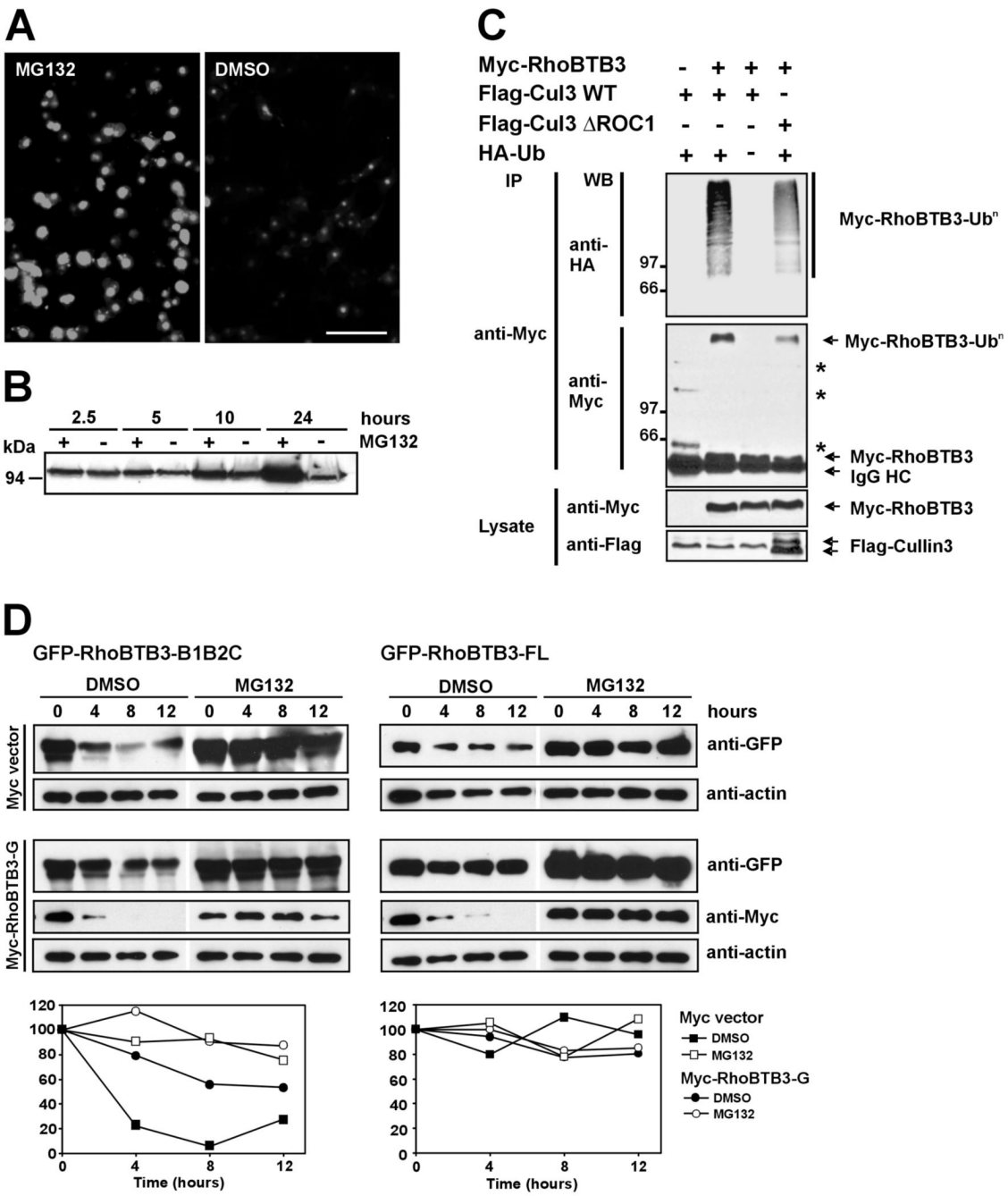


**Figure 5. Degradation of RhoBTB3 in the 26S proteasome**

(**A**) COS7 cells were transfected with GFP-tagged RhoBTB3 and treated with the proteasomal inhibitor MG132 (5 μM) or DMSO as a control. After 24 hours treated cells showed a clear accumulation of the fusion protein. Bar represents 100 μm. (**B**) COS7 cells were transfected with GFP-tagged RhoBTB3 and treated with the proteasomal inhibitor MG132 (10 μM) or DMSO as a control. Homogenates were collected at different time points and subjected to Western blotting. GFP-tagged RhoBTB was detected with an antibody against GFP. (**C**) *In vivo* Cul3-dependent ubiquitinylation of RhoBTB3. 293T cells were transfected with the indicated plasmids. Flag-Cul3 ΔROC1 is a ROC1 binding deficient mutant of Cul3. 20 hours after transfection cells were treated with 25 μM MG132 for 4 h prior to cell lysis. Lysates were

immunoprecipitated with anti-Myc antibody and resolved by SDS-PAGE, followed by immunoblotting with anti-HA. The same membrane was stripped and probed with anti-Myc antibody. The asterisk indicates a nonspecific protein precipitated by the anti-Myc antibody. Lysates were analyzed for the presence of the corresponding ectopically expressed proteins. (**D**) Intramolecular interaction prevents degradation of RhoBTB3. 293T cells were transfected with the indicated GFP and Myc-tagged proteins. See Fig. 1A for nomenclature of the constructs. 16 hours after transfection cells were split and treated with 100 μM cycloheximide to arrest protein biosynthesis. As a control, proteasomal degradation was inhibited with 10 μM MG132. Lysates were prepared at the indicated time points and analyzed for the amounts of Myc and GFP-tagged protein. The blots were reprobed for actin as a loading control. The graphs show the amount of the corresponding GFP fusion protein normalized to the amount of actin and expressed as percentage relative to the 0 time point. Co-transfection of the GTPase domain leads to an accumulation of the B1B2C region of RhoBTB3 but has little effect on the full length protein, which is more stable.

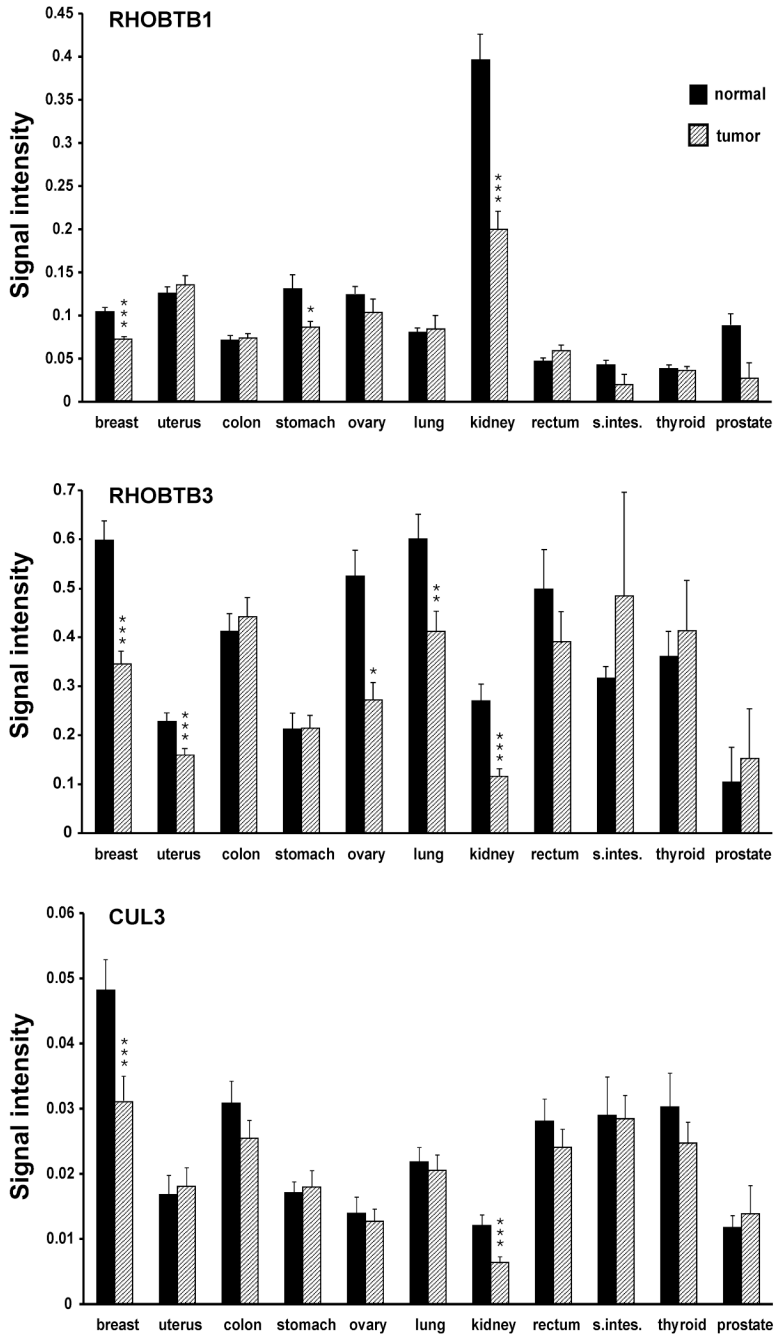


**Figure 6. Expression of *RHOBTB1*, *RHOBTB3* and *CUL3* in matched normal versus tumor tissues**

The data was derived by quantification of the blots in Suppl. Figure 4 using a Phosphorimager and ImageQuant software. The background-adjusted volumes for each sample were normalized with the corresponding background-adjusted ubiquitin probe. Mean volumes ± SEM (standard error of the mean) are shown. Asterisks indicate statistical significance by two-way paired Student's t-test (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$). Samples of cervix and pancreas, each represented by one tissue sample, were omitted from the analysis. S. intes, small intestine.

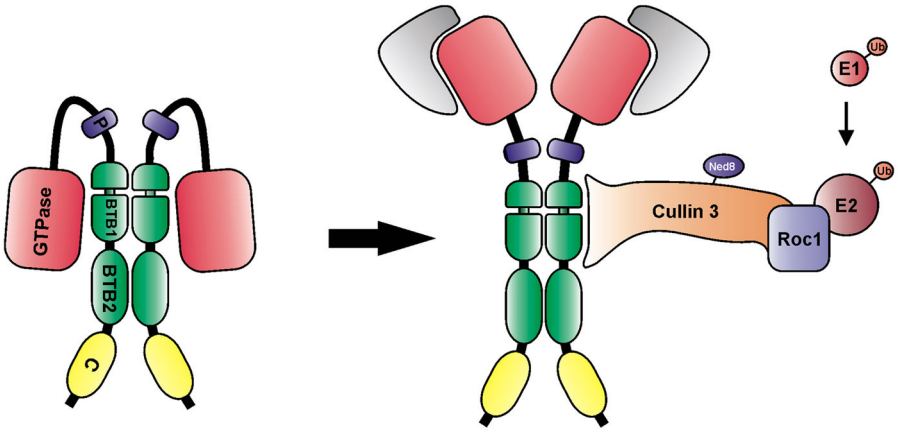


**Figure 7. Model of the mechanism of action of RhoBTB proteins**

RhoBTB proteins recruit Cul3 (regulated by attachment of Ned8), Roc and the E2 (ubiquitin conjugating enzyme) component of the ubiquitinylation pathway to constitute an ubiquitin ligase. E1 is the ubiquitin-activating enzyme. RhoBTB proteins probably exist as homo and heterodimers. The first BTB domain is interrupted by an insertion of variable length amongst the three RhoBTB isoforms. RhoBTB is depicted as a parallel dimer, but it remains to be established whether the dimer is parallel or antiparallel. An intramolecular interaction between the GTPase domain and the BTB region would maintain the dimer in an inactive state. Although we have depicted the GTPase interacting with the BTB tandem of the same molecule, an interaction with the tandem of the partner molecule is equally possible. Interaction of the GTPase domain with unknown proteins would relieve the autoinhibition, allowing recruitment of the Cul3 scaffold to the first BTB domain. We cannot exclude at this moment that the GTPase acts by hindering ubiquitinylation by, rather than binding to, the Cul3-dependent complex. The GTPase and other domains, as well as the insertion of the first BTB domain could function as substrate recognition domains. The proline-rich region is a potential SH3 domain-binding domain. Note also that RhoBTB proteins become ubiquitinylated. The substrates may be involved in regulating cell growth and vesicle trafficking, and would be kept at low levels by degradation in the 26S proteasome. Reduced expression of RhoBTB or mutations that impair formation of the complex would result in accumulation of the substrates and contribute to tumor formation or progression.

**Table 1**

Correlation analysis of the change of expression levels of RHOBTB3 vs. CUL3, RHOBTB1 vs. CUL3 and RHOBTB1 vs. RHOBTB3. . For a given gene the relative expression change between a tumor sample and its paired normal tissue was calculated from the normalized background-adjusted volumes obtained after quantification of the cancer profiling array of Suppl. Fig. 4. The correlation of the relative expression changes was calculated pairwise as indicated in the table, either for each tissue or for the whole population. Tissues represented by six or less samples were not considered individually.

| | | RHOBTB3 vs. CUL3 | | RHOBTB1 vs. CUL3 | | RHOBTB1 vs. RHOBTB3 | |
|---|---|---|---|---|---|---|---|
| **Tissue** | **n** | **r** | **P** | **r** | **P** | **r** | **P** |
| Breast | 50 | 0.6235 | *** | 0.4507 | *** | 0.1215 | |
| Uterus | 42 | 0.5486 | *** | 0.5691 | *** | 0.3804 | * |
| Colon | 35 | 0.0924 | | −0.0752 | | 0.5785 | *** |
| Stomach | 27 | 0.1258 | | 0.7467 | *** | 0.0530 | |
| Ovary | 14 | 0.0857 | | −0.3249 | | 0.3472 | |
| Lung | 21 | 0.4982 | * | 0.4157 | | 0.1869 | |
| Kidney | 20 | 0.0507 | | −0.0271 | | −0.1906 | |
| Rectum | 18 | 0.4936 | * | 0.1325 | | 0.2267 | |
| All | 237 | 0.3390 | *** | 0.1526 | ** | 0.2892 | *** |

r, correlation coefficient.

Asterisks indicate statistical significance by a test of independence (*** P < 0.001; ** P < 0.01; * P < 0.05).