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Hypertension-causing Mutations in Cullin3 Protein Impair RhoA Protein Ubiquitination and Augment the Association with Substrate Adaptors*

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Background: Cullin3 ubiquitin ligase regulates protein turnover by promoting the ubiquitination of substrates.
Results: Ubiquitination of RhoA is impaired by mutations in Cullin3.
Conclusion: Disease-causing Cullin3 mutations impair the turnover of RhoA protein and may sequester substrates adaptors.
Significance: Mutations in Cullin3 cause reduced ubiquitination and elevation of RhoA levels, which may enhance RhoA and Rho kinase signaling in a variety of cell types and could potentially contribute to hypertension.

Cullin-Ring ubiquitin ligases regulate protein turnover by promoting the ubiquitination of substrate proteins, targeting them for proteasomal degradation. It has been shown previously that mutations in Cullin3 (Cul3) causing deletion of 57 amino acids encoded by exon 9 (Cul3Δ9) cause hypertension. Moreover, RhoA activity contributes to vascular constriction and hypertension. We show that ubiquitination and degradation of RhoA is dependent on Cul3 in HEK293T cells in which Cul3 expression is ablated by either siRNA or by CRISPR-Cas9 genome editing. The latter was used to generate a Cul3-null cell line (HEK293T^{Cul3KO}). When expressed in these cells, Cul3Δ9 supported reduced ubiquitin ligase activity toward RhoA compared with equivalent levels of wild-type Cul3 (Cul3WT). Consistent with its reduced activity, binding of Cul3Δ9 to the E3 ubiquitin ligase Rbx1 and neddylation of Cul3Δ9 were impaired significantly compared with Cul3WT. Conversely, Cul3Δ9 bound to substrate adaptor proteins more efficiently than Cul3WT. Cul3Δ9 also forms unstable dimers with Cul3WT, disrupting dimers of Cul3WT complexes that are required for efficient ubiquitination of some substrates. Indeed, coexpression of Cul3WT and Cul3Δ9 in HEK293T^{Cul3KO} cells resulted in a decrease in the active form of Cul3WT. We conclude that Cul3Δ9-associated ubiquitin ligase activity toward RhoA is impaired and suggest that Cul3Δ9 mutations may act dominantly by sequestering substrate adaptors and disrupting Cul3WT complexes.

Cullin-Ring ubiquitin ligase (CRL)[2] complexes form the largest class of multicomponent E3 ubiquitin ligases and are evolutionally conserved. CRL complexes target a wide array of proteins involved in a variety of diverse biological processes and promote their ubiquitination and degradation by the 26S proteasome (1). The CRL3 complex is assembled around Cullin3 (Cul3), which acts as a scaffold that links a RING E3 ubiquitin ligase protein at its C terminus with a variable BTB-containing substrate adaptor at its N terminus (2, 3). Cul3 plays an important role in regulating arterial pressure, and loss of its ubiquitin ligase activity is linked to hypertension, which is attributable to increased RhoA protein in vascular smooth muscle cells (4). Cul3 is reported to regulate RhoA turnover by directly interacting with a BTB domain-containing protein, Bacurd1, which acts as a substrate adaptor to recruit RhoA to the CRL3 complex (5). Genetic evidence suggests that the Cul3 pathway may be an important regulator of blood pressure (6). Mutations in either Cul3 or KLHL3, a Cul3 adaptor protein, were discovered in patients with pseudohypoaldosteronism type II (PHAII) syndrome, characterized by hypertension, metabolic acidosis, and hyperkalemia (6). Impairment of Cul3-KLHL3 complexes resulted in the accumulation of WNK4 because of a loss of its ubiquitination and degradation, therefore impairing the regulation of renal sodium and potassium (7–10). It is notable that all identified mutations in Cul3 resulted in an in-frame deletion of exon 9, leading to aberrant splicing of exons 8 and 10 with loss of 57 amino acids (403–459), which we have termed Cul3Δ9. These Cul3Δ9 mutations were found to be dominant and *de novo* (6). Importantly, over 90% of patients with Cul3Δ9 mutations develop early-onset hypertension.

Despite reports demonstrating an important role for Cul3-mediated regulation of renal function, the mechanism by which Cul3 functions in other cell types, such as the vasculature, remains unclear. We have reported previously that pharmacologically inhibiting the Cullin pathway increased agonist-induced vascular contraction through a RhoA/Rho kinase (ROCK)-dependent mechanism *ex vivo* and hypertension *in vivo* (4). A common feature of hypertension is increased ROCK activity, and blocking ROCK signaling lowered the blood pressure in hypertensive subjects (11–13). To determine whether the Δ9 mutation in Cul3 could affect this regulatory pathway, we assessed the activities of wild-type Cul3 (Cul3WT) and Cul3Δ9 expressed in HEK293T cells. We tested whether RhoA

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[2] The abbreviations used are: CRL, Cullin-Ring ubiquitin ligase; PHAII, pseudohypoaldosteronism type II; ROCK, RhoA/Rho kinase; IP, immunoprecipitation.

ubiquitination was dependent upon Cul3 using siRNA-mediated ablation and CRISPR-Cas9 genome editing and whether the Cul3Δ9 mutation altered RhoA ubiquitination and degradation or the composition of CRL3 complexes. Our results show that the hypertension-causing Cul3Δ9 mutant exhibited impaired ubiquitination of RhoA compared with wild-type Cul3 (Cul3WT). Consistent with its reduced activity, neddylation of Cul3Δ9 and its binding to the requisite E3 ubiquitin ligase, Rbx1, were impaired compared with Cul3WT. Conversely, Cul3Δ9 exhibited augmented binding to Bacurd1 and two other BTB domain proteins we tested. Cul3Δ9 also formed heterodimeric complexes with Cul3WT. Dimerization of CRL complexes is required for efficient ubiquitination of some substrates (14–16), and Cul3WT formed stable homodimers. By contrast, heterodimers between Cul3Δ9 and Cul3WT were dramatically stabilized only in the presence of proteasome and CRL inhibitors. These results suggest that defects in Cul3Δ9-associated ubiquitin ligase activity impair RhoA ubiquitination and stabilize RhoA protein by disrupting wild-type CRL3 complexes. Combined with our previous study, these findings also suggest that wild-type Cul3 contributes to blood pressure regulation by tightly modulating the ubiquitination and, therefore, the stability of RhoA.

Experimental Procedures

Cell Culture, Transfection, and siRNA Gene Silencing—HEK293T cells were obtained from the ATCC and maintained in high-glucose DMEM supplemented with 10% FBS and 100 units/ml penicillin and streptomycin at 37 °C in a 5% CO_2 incubator. Cells were subcultured 24 h prior to transfection. Transfections of plasmids and siRNAs were performed using Lipofectamine LTX (Invitrogen) according to the protocol of the manufacturer. Non-targeting sequence siRNA or siRNA targeting exon 9 of human Cul3, which is deleted in Cul3Δ9 (Integrated DNA Technologies) were used. Where indicated, HEK293T cells were treated with 5 μM MG132 (Cayman) or 1 mM MLN4924 (Cayman) for 16 h prior to lysis. Cells were harvested 72 h post-transfection, and total protein lysates were stored at −80 °C until further analysis.

Generation of Cul3-edited HEK293T Cells by CRISPR-CAS9—The plasmid vector expressing Cas9 enzyme driven by the CMV promoter and an enhanced GFP-selectable marker was obtained from OriGene (plasmid GE100018, Rockville, MD). CRISPR guide RNA specifically targeting exon 7 of Cul3 was cloned into the vector (forward gRNA AGTTCCTATTTGAGGGAGCA and complement strand TGCTCCCTCAAATAGGAACT) and verified by DNA sequencing. HEK293T cells were transiently transfected using X-tremeGENE 9 DNA transfection reagent (Roche Diagnostics), and positive cells were sorted for enhanced GFP by flow cytometry. One week after transfection, single clones were isolated by serial dilution. The region encompassing the Cul3 exon 7 locus was PCR-amplified, and the product was cloned into a pCRII-TOPO vector and sequenced. Sequencing revealed deletion of 8 bp (chromosome 2: 224,506,917–224,506,910) and 111 bp (chromosome 2: 224,506,934–224,506,824) on each allele, respectively. The efficiency of Cul3 ablation was verified by immunoblotting.

Plasmids and Site-directed Mutagenesis—Cul3WT and RhoBTB1 ORF cDNAs were amplified from mouse lung cDNA by PCR and cloned into the pCR 2.1-Topo vector (Invitrogen). To obtain Cul3Δ9 cDNA, splicing by overhang extension PCR was used to delete exon 9 in Cul3WT. RhoBTB1, Cul3WT, and Cul3Δ9 cDNAs were then transferred into the pCMV6-AN-MYC and pCMV6-AN-DDK vectors (OriGene). Rbx1 cDNA was amplified from a HEK293T cDNA library and cloned into the pCMV6-AN-MYC vector. Plasmids were verified by DNA sequencing. The GFP-RhoA WT and GFP-RhoA T19N plasmids were obtained from Addgene (plasmids 12965 and 12967, respectively). Human KLHL3 ORF (Promega) and mouse Bacurd1 ORF (OriGene) were cloned into pCMV6-AN-HIS (OriGene). Myc-Ubiquitin and HA-Cul3ΔRbx1 were gifts from Dr. Fred Quelle (17) and Dr. Stefan Strack (18), respectively.

To obtain an siRNA-resistant variant of Cul3WT (Cul3WTR), site-directed mutagenesis was performed on FLAG-Cul3WT using a PCR-based strategy with the QuikChange Lightning multi-site directed mutagenesis kit (Agilent Technologies). Briefly, mutagenic primers were designed using the Agilent QuikChange primer design program. Primers were designed from Cul3 coding sequence cDNA with changes of A to G at nucleotide 1218, A to G at nucleotide 1221, and A to T at nucleotide 1224. This mutagenesis does not alter the original protein coding sequence of Cul3WT, which was verified by sequencing.

Antibodies and Drugs—Antibodies used in this study were as follows: Cul3 (Bethyl, catalog no. A301-109A), FLAG (Sigma, catalog no. F1804), Myc (Cell Signaling Technology, catalog no. 9B11), HA (eBioscience, catalog no. 14-6756), Rbx1 (Abcam, catalog no. ab86862), Nedd8 (Cell Signaling Technology, catalog no. 19E3), RhoA (Cell Signaling Technology, catalog no. 67B9), Myc-agarose beads (Santa Cruz Biotechnology, catalog no. sc-40 ac), HA-agarose beads (Santa Cruz Biotechnology, catalog no. sc-7392 ac), GFP (Santa Cruz Biotechnology, catalog nos. sc-9996 and sc-8334), GAPDH (Santa Cruz Biotechnology, catalog no. sc-32233), and His (OriGene, catalog no. TA100013). MG132 (Cayman) and MLN4924 (Active-Biochem) were dissolved in dimethyl sulfoxide.

Immunoprecipitation and Western Blotting—Transfected HEK293T cells were lysed in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS in 1× PBS with 1× proteinase inhibitor mixture). Cell lysates were centrifuged at 4 °C, and total protein lysate was quantified using the Lowry assay. 1 mg of total protein lysate was incubated with either 2 μg of primary antibody or control IgG antiserum for 2 h at 4 °C, followed by incubation with 50 μl of TrueBlot anti-mouse or anti-rabbit Ig IP bead slurry (Rockland Immunochemicals) overnight. Beads were washed four times with lysis buffer, and immunoprecipitates were eluted for 10 min at 100 °C in 50 μl of 2× sample buffer. Protein was separated by SDS-PAGE and transferred to an Immobilon-P membrane. The membrane was immunoblotted with primary antibody followed by EasyBlot anti-mouse IgG (HRP, GeneTex) or protein A-HRP conjugate (Bio-Rad), and protein bands were detected using ECL (Amersham Biosciences).

Purification of Recombinant Proteins—Escherichia coli strain BL21 (DE3) harboring GST or GST-RhoA-T19N expression

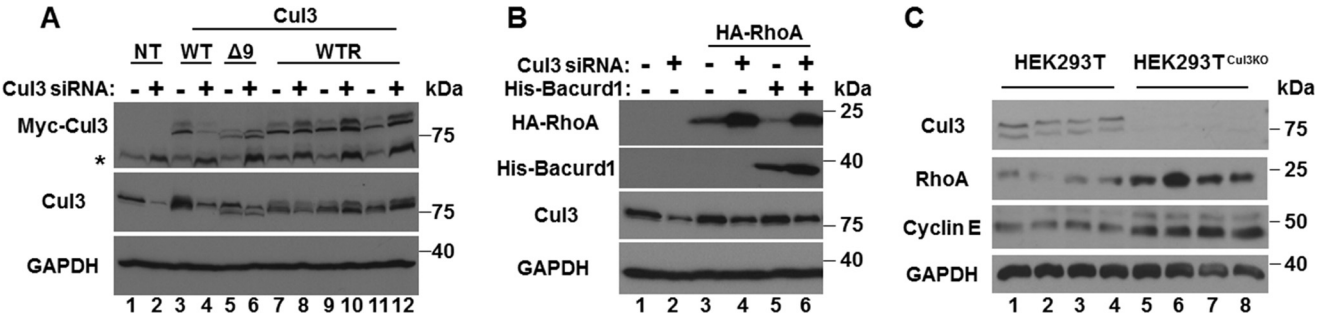

FIGURE 1. **Knockdown of Cul3 leads to increased RhoA protein expression.** *A*, immunoblot validating siRNAs targeting endogenous Cul3 but not Cul3Δ9 or resistant Cul3WT (*Cul3WTR*). *B*, immunoblot analyses showing that turnover of RhoA protein is Cul3-dependent and is more efficient in the presence of Bacurd1. HEK293T cells were cotransfected with plasmids expressing His-Bacurd1, HA-RhoA, or both with/without siRNA targeting endogenous Cul3. Cells lysates were isolated 72 h post-transfection. *C*, immunoblot showing that CRISPR-Cas9 genome editing of Cul3 increases RhoA and Cyclin E protein expression in HEK293T^{Cul3KO} cells. The *asterisk* indicates a nonspecific band. The position of each molecular weight size marker (in kilodaltons) is shown. Representative blots of three experiments each *B* and C are shown. *NT*, non-transfected.

plasmids was grown in ampicillin-containing LB medium overnight. The next day, the culture was diluted with ampicillin-containing Luria broth (LB) medium and incubated for an additional 30 min at 37 °C. Protein expression was induced for 16 h at 22 °C with 0.1 mM isopropyl-β-D-thiogalactopyranoside. *E. coli* cultures expressing GST or GST-RhoA-T19N were harvested by centrifugation at 4 °C and resuspended in lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, and 1% Triton X-100. Prior to using the lysis buffer, 1 mM DTT, 1 mM PMSF, and protease inhibitors were added. GST fusion proteins were purified using glutathione-Sepharose 4B beads (GE Healthcare) and eluted from the beads with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0).

In Vivo and in Vitro Ubiquitination Assays—For *in vivo* ubiquitination, experiments were performed as described previously (19). Briefly, transfected cells were treated with 2 μM MG132 and/or 1 μM MLN4924 for 16 h prior to lysis. Cells were lysed in lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0)) supplemented with 25 mM *N*-ethylmaleimide, 25 μM MG132, and a protease inhibitor mixture tablet (Roche). Lysates were boiled for 10 min to release bound proteins and sonicated immediately. Sonicated lysates were further diluted with dilution buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, and 1% Triton). 1 mg of total protein lysate was used for immunoprecipitation as described above. Beads were washed four times with wash buffer (10 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, 1% Nonidet P-40) supplemented with 25 mM *N*-ethylmaleimide and a protease inhibitor mixture tablet. The respective antibodies were used for detection of ubiquitinated proteins by Western blotting.

For *in vitro* ubiquitination, recombinant GST or GST-RhoA-T19N was expressed in *E. coli* and purified as described. The Cul3-Bacurd1-Rbx1 complex was purified by immunoprecipitation with Cul3 antibody from 1 mg of lysates of HEK293T cells transfected with His-Bacurd1. Precipitates were washed four times with 50 mM NaCl, 50 mM HEPES, 10% glycerol, 0.1% Tween 20, and 20 mM Tris (pH 7.5). Ubiquitination reaction mixture contained the purified CRL3 complex bound on beads, 250 ng of E1 (Boston Biochem), 500 ng of E2 (UbcH5A, Boston Biochem), 12 μg of Myc-ubiquitin (Boston Biochem), 5 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 10 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase (Sigma), 1 μM ubiquitin aldehyde (Boston Biochem), and 20 μg of purified GST-RhoA-T19N or GST protein. The reactions were diluted to a final volume of 1 ml in 50 mM HEPES buffer (pH 7.5) and incubated for 2 h at 37 °C on an end-over-end rotator. Reaction products were sedimented by centrifugation at 500 × *g* for 5 min, and the supernatant was precipitated with glutathione-Sepharose 4B beads for 1 h at 4 °C. Precipitated proteins were then separated by SDS-PAGE, and ubiquitinated proteins detected by Western blot analysis with anti-myc antibody.

Statistical Analyses—All data are expressed as mean ± S.E. Data were analyzed using paired Student's *t* test or one-way analysis of variance, followed by Tukey post hoc test for one-way analysis of variance where appropriate. Statistical significance was accepted at $p < 0.05$.

Results

RhoA Turnover Is Cul3-dependent and More Efficient in the Presence of Bacurd1—We first confirmed that Cul3 mediates RhoA protein turnover (5). Because endogenous Cul3 protein is highly expressed in HEK293T cells, siRNA targeting exon 9 was designed to knock down endogenous Cul3 so that the activity of transfected Cul3WT and Cul3Δ9 could be examined without interference by endogenous Cul3. We then performed mutagenesis of the Cul3 cDNA to generate a variant of Cul3WT (called Cul3WTR) that is resistant to the siRNA. The efficacy and specificity of this siRNA was validated by immunoblot, showing that the siRNA was effective in knocking down endogenous Cul3 but not the transfected Cul3WTR or Cul3Δ9 constructs (Fig. 1*A*). SiRNA-mediated knockdown of endogenous Cul3 was achieved in HEK293T cells expressing HA-RhoA with or without overexpression of its substrate recognition adaptor, Bacurd1. Loss of Cul3 increased RhoA (Fig. 1*B*, compare *lanes 3* and *4*), and the presence of Bacurd1 facilitated RhoA turnover (Fig. 1*B*, compare *lanes 3* and *5*). This Bacurd-induced facilitation was dependent on Cul3 because Cul3 siRNA blocked the decrease in RhoA (Fig. 1*B*, compare *lanes 5* and *6*). We next employed CRISPR/Cas9 genome editing to generate cells lacking endogenous Cul3, HEK293T^{Cul3KO} cells (Fig. 1*C*). The level of Cul3 protein was clearly ablated, whereas the levels of the Cul3 substrates RhoA and Cyclin E were increased substantially in HEK293T^{Cul3KO} cells compared with Cul3-expressing HEK293T cells (Fig. 1*C*).

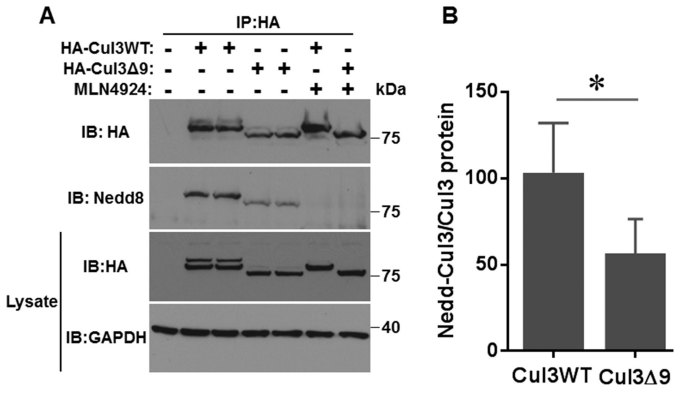

FIGURE 2. **Cul3Δ9 neddylation is impaired.** *A*, immunoblot (IB) analyses of HEK293T^{Cul3KO} cells transfected with HA-Cul3WT or HA-Cul3Δ9 and treated with either 1 μM MLN4924 or dimethyl sulfoxide. The immunoprecipitation experiment was performed as described under "Experimental Procedures." The primary antibody used for each IP is indicated. The position of each molecular weight size marker (in kilodaltons) is shown. *B*, quantification of neddylated Cul3 normalized to immunoprecipitated HA-Cul3. *, $p < 0.05$ neddylated-Cul3WT *versus* neddylated-Cul3Δ9. *Error bars* represent mean ± S.E. ($n = 3$).

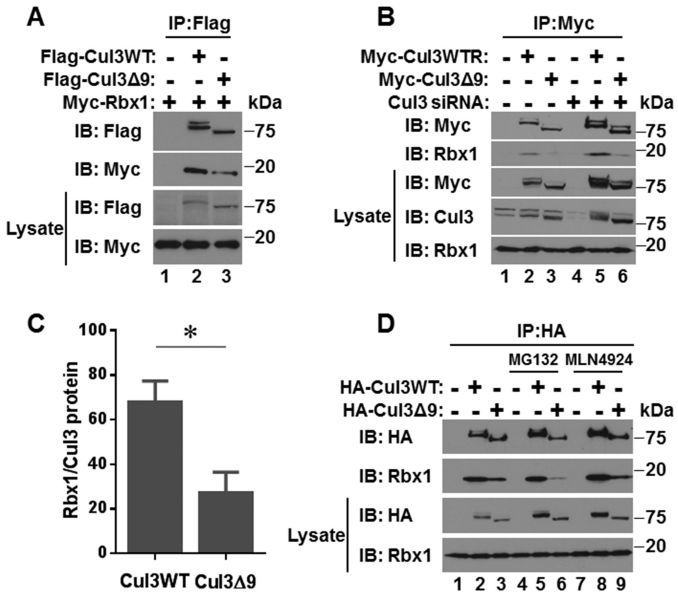

FIGURE 3. **Cul3Δ9 exhibits impaired binding to Rbx1.** *A*, immunoblot (*IB*) analyses of HEK293T cells expressing FLAG-Cul3WT or FLAG-Cul3Δ9 cotransfected with Myc-Rbx1 and subjected to IP. *B*, HEK293T cells were transfected with Myc-Cul3WT or Myc-Cul3Δ9. Where indicated, siRNA targeting endogenous Cul3 was also transfected. *C*, quantification of immunoprecipitated endogenous Rbx1 normalized to immunoprecipitated Myc-Cul3WT or Myc-Cul3Δ9. *Error bars* represent mean ± S.E. ($n = 4$). *D*, immunoblot analyses of HEK293T^{Cul3KO} cells transfected with HA-Cul3WT or HA-Cul3Δ9 and treated with/without 2 μM MG132 or 1 μM MLN4924 for 16 h prior to lysis. Lysates indicate samples not subjected to immunoprecipitation as controls for antibody specificity. The position of each molecular weight size marker (in kilodaltons) is shown. Representative blots of three experiments each for *A* and *D* are shown. *, $p < 0.05$ Rbx1 immunoprecipitated with Myc-Cul3WT *versus* Myc-Cul3Δ9.

Neddylation of Cul3Δ9 and Binding to the E3 Ubiquitin Protein Ligase Rbx1 Are Impaired—The ubiquitin ligase activity of the CRL complex depends on the addition of a Nedd8 moiety onto Cullin proteins, a posttranslational modification known as neddylation (20–22). Therefore, we determined whether Cul3Δ9 can be neddylated efficiently by probing immunoprecipitated FLAG-Cul3WT or FLAG-Cul3Δ9 with Nedd8 antibody. Both Cul3WT and Cul3Δ9 were neddylated, indicating that Cul3Δ9 can be neddylated (Fig. 2*A*). However, the relative amount of neddylation was reduced significantly in immunoprecipitates of Cul3Δ9 compared with Cul3WT (Fig. 2*B*), suggesting that neddylation is impaired by the Cul3Δ9 mutation. Certainly, there was no evidence for enhancement in neddylation of Cul3Δ9, as reported previously (23). Specific detection of neddylated forms of Cul3 was confirmed by their absence in Cul3 immunoprecipitates from cells treated with MLN4924, a Nedd8-activating enzyme inhibitor that prevents all forms of neddylation. MLN4924 also acts as a pan-CRL inhibitor by preventing neddylation of Cullins. Consistent with reports that neddylated Cullins are less stable than non-neddylated forms (24), there was an increase in the abundance of both Cul3WT and Cul3Δ9 in the lysates of cells treated with MLN4924.

We next considered the structure of the CRL3 complex. Cul3 links BTB domain-containing adaptors with Rbx1, a RING E3 ubiquitin ligase protein. Rbx1 recruits E2-conjugating enzymes that directly transfer ubiquitin to substrates bound to the adaptors. Indeed, the 57 amino acids deleted in Cul3Δ9 overlap with the Cullin homology domain required for Rbx1 binding to Cul3 (25–30). To examine Cul3Δ9 binding to Rbx1, FLAG-Cul3WT or FLAG-Cul3Δ9 cotransfected with Myc-Rbx1 in HEK293T cells was analyzed by immunoprecipitation and Western blotting using the FLAG and Myc antibodies, respectively. Immunoprecipitation for the FLAG epitope revealed an impairment of Myc-Rbx1 binding to FLAG-Cul3Δ9 compared with FLAG-Cul3WT (Fig. 3*A*). To gain insight into the association of endogenous Rbx1 with Cul3Δ9, we deleted endogenous Cul3 using siRNA in HEK293T cells expressing Myc-Cul3WTR or Myc-Cul3Δ9. Immunoblotting of endogenous Rbx1 after Cul3 immunoprecipitation with Myc antibody shows that Cul3Δ9 exhibits impaired binding to Rbx1 compared with Cul3WT (Fig. 3, *B*, *lanes 2 and 3*, and *C*). To rule out the possibility of enhanced turnover of Rbx1 in the presence of Cul3Δ9, HEK293T cells expressing HA-Cul3WT or HA-Cul3Δ9 were treated with either the proteasome inhibitor MG132 or the neddylation inhibitor MLN4924. Neither MG132 nor MLN4924 improved Rbx1 binding to Cul3Δ9 (Fig. 3*D*). Because Rbx1 is also the Nedd8 ligase responsible for neddylation of Cul3, the reduced association between Cul3Δ9 and Rbx1 is consistent with the reduced neddylation of Cul3Δ9. Functionally, impaired binding of Cul3Δ9 to Rbx1 may also impair the ubiquitination of substrates recruited to CRL3 complexes containing Cul3Δ9.

Defects in Cul3Δ9 Ubiquitin Ligase Activity Impair RhoA Ubiquitination—Next we determined whether Cul3Δ9 could support ubiquitination activity. To test this, we utilized RhoA-T19N, a dominant-negative RhoA mutant, which mimics the conformation of RhoA in its inactive GDP-bound state (31, 32). Bacurd1 preferentially binds to RhoA-GDP rather than RhoA-GTP, thereby reducing the pool of RhoA-GDP available for activation by Rho guanine nucleotide exchange factors (RhoGEFs) (5). *In vitro* ubiquitination studies revealed that the Cul3 complex is sufficient to mediate ubiquitination of RhoA (Fig. 4*A*). To confirm that Cul3 directly regulates RhoA stability *in vivo*, cells transiently expressing GFP-RhoA-T19N and

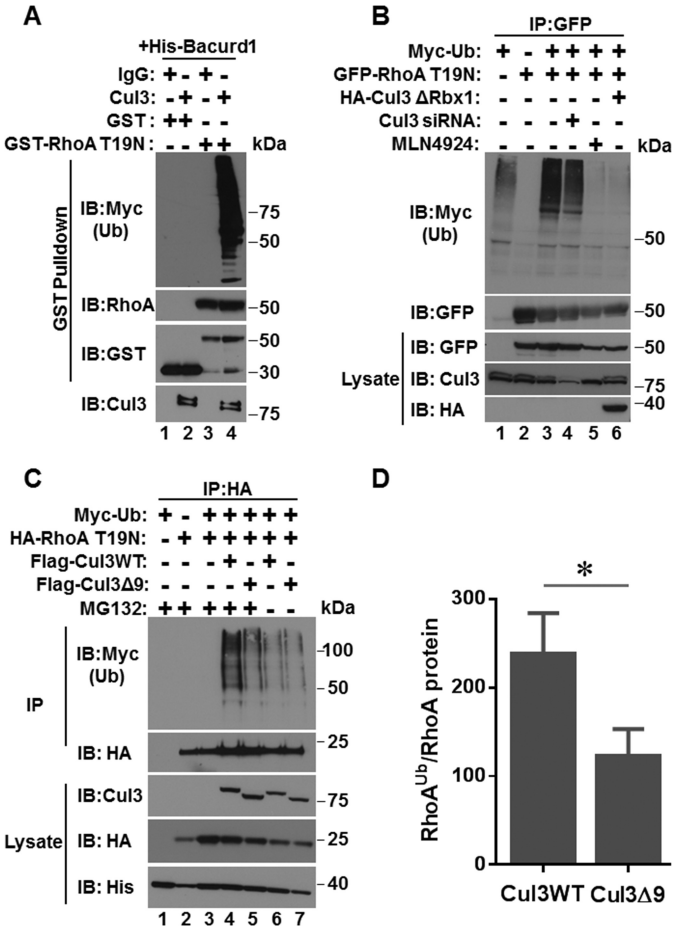

FIGURE 4. **Defects in Cul3Δ9 ubiquitin ligase activity impair RhoA ubiquitination.** *A*, immunoblot (*IB*) analysis of *in vitro* ubiquitination of RhoA by the Cul3/Bacurd1 complex. Cul3/Bacurd1 complexes were isolated by immunoprecipitating Cul3 from HEK293T cell transfected with His-Bacurd1. IP with control IgG antibody was performed in cells expressing His-Bacurd1. Cul3/Bacurd1 complexes and recombinant GST or GST-RhoA T19N were used in an ubiquitination (*Ub*) assay as described. *B*, immunoblotting assay showing that *in vivo* RhoA ubiquitination is blunted by Cul3 siRNA and is blocked by dominant negative Cul3 or MLN4924. GFP-RhoA T19N was cotransfected with Myc-Ub or empty Myc vector in HEK293T cells and incubated with 2 μM MG132. Where indicated, cells were transfected with Cul3 siRNA or HA-Cul3ΔRbx1 or treated with 1 μM MLN4924. GFP immunoprecipitates were probed with anti-Myc to reveal ubiquitin conjugates of GFP-RhoA. Representative blots of three experiments are shown. *C*, immunoblotting assay showing defects of Cul3Δ9 on *in vivo* ubiquitination of RhoA. HA-RhoA T19N was cotransfected with Myc-Ub or empty Myc vector along with FLAG-Cul3WT or FLAG-Cul3Δ9 and His-Bacurd1 and incubated with or without 2 μM MG132 in HEK293T^{Cul3KO} cells. HA immunoprecipitate was probed with anti-Myc to reveal ubiquitin conjugate of HA-RhoA. *D*, quantification of ubiquitinated RhoA (*RhoA^{Ub}*) normalized to immunoprecipitated HA-RhoA T19N. *Error bars* represent mean ± S.E. ($n = 3$). The position of each molecular weight size marker (in kilodaltons) is shown. *, $p < 0.05$ ubiquitinated RhoA in the presence of FLAG-Cul3WT *versus* FLAG-Cul3Δ9.

Myc-Ub were treated with MG132 prior to lysis. GFP-RhoA-T19N was immunoprecipitated under denaturing conditions and analyzed for modification by ubiquitin. Ubiquitin-modified RhoA-T19N (RhoA^{ub}) was detected by immunoblotting with Myc antibody (Fig. 4*B*, *lane 3*). We confirmed that ubiquitination of RhoA was dependent on Cul3 because it was decreased with Cul3 siRNA (Fig. 4*B*, *lane 4*) and ablated by either MLN4924 (Fig. 4*B*, *lane 5*) or transfection with a dominant negative Cul3 mutant (Cul3-ΔRbx1) that does not bind Rbx1 (Fig. 4*B*, *lane 6*).

We next tested whether Cul3Δ9 exhibited ubiquitin ligase activity toward RhoA *in vivo*. HEK293T^{Cul3KO} cells were transfected with constructs expressing HA-RhoA-T19N and/or Myc-Ub and FLAG-Cul3WT or FLAG-Cul3Δ9. Transfected cells were treated with MG132 or vehicle 16 h prior to immunoprecipitation of HA-RhoA-T19N under denaturing conditions (Fig. 4*C*). We observed the accumulation of polyubiquitinated RhoA upon treatment with MG132 in cells expressing Cul3WT (Fig. 4*C*, *lane 4*), but this was reduced significantly in cells expressing Cul3Δ9 (Fig. 4, *C*, *lane 5*, and *D*). The accumulation of polyubiquitinated RhoA was reduced in cells that lacked MG132 treatment, consistent with turnover of RhoA^{ub} by the proteasome (Fig. 4*C*, *lanes 6* and *7*). Consistent with reduced binding with Rbx1, these data suggest that Cul3Δ9 exhibits a significant impairment in ubiquitin ligase activity toward RhoA.

Cul3Δ9 Binds to Substrate Adaptors More Efficiently Than Cul3WT—The data presented above suggest that Cul3Δ9 exhibits impaired ubiquitination activity, at least toward RhoA. We next wanted to understand how this mutation may act dominantly. We therefore sought to examine the association of Cul3Δ9 with substrate adaptor proteins. Cul3 recruits several BTB domain-containing proteins that confer substrate specificity for Cul3-dependent ubiquitination (reviewed in Ref. 2). To assess the association of Cul3Δ9 with substrate adaptors, FLAG-Cul3WT or FLAG-Cul3Δ9 was cotransfected with various epitope-tagged Cul3 adaptors in HEK293T cells. Immunoprecipitation with FLAG antibody showed an interaction of both Cul3WT and Cul3Δ9 with His-Bacurd1 (Fig. 5*A*), Myc-RhoBTB1 (Fig. 5*B*), and His-KLHL3 (Fig. 5*C*) (all quantified in Fig. 5*D*). Interestingly, unlike the impaired interaction with Rbx1, all three adaptor proteins exhibited enhanced binding to Cul3Δ9 compared with Cul3WT. Because some Cul3 substrate adaptor proteins are themselves ubiquitinated through their association with Cul3:Rbx1 (10, 33, 34), we sought to determine whether the increase in substrate adaptors binding to Cul3Δ9 was due to impaired degradation of adaptor proteins by Cul3Δ9. HEK293T^{Cul3KO} cells coexpressing FLAG-Cul3WT or FLAG-Cul3Δ9 with His-Bacurd1 were treated with MG132 or MLN4924. Immunoprecipitation of FLAG and immunoblotting for His-Bacurd1 still showed enhanced binding of Bacurd1 with Cul3Δ9 in MG132 and MLN4924-treated HEK293T^{Cul3KO} cells compared with Cul3WT (Fig. 5*E*). These data strongly suggest that enhanced binding of Cul3Δ9 to Bacurd1 is not due to a decrease in its degradation when associated with Cul3Δ9 compared with Cul3WT. Instead, Cul3Δ9 may act dominantly by sequestering adaptors from Cul3WT, therefore interfering with the ubiquitin ligase activity of Cul3WT.

To determine whether Cul3Δ9 affects the availability of adaptor proteins, we investigated the levels of select adaptor proteins in the presence of Cul3Δ9 *versus* Cul3WT. We transiently expressed His-Bacurd1 or Myc-RhoBTB1 in HEK293T cells with FLAG-Cul3WT or FLAG-Cul3Δ9. The level of Myc-RhoBTB1 protein was decreased by FLAG-Cul3WT but not FLAG-Cul3Δ9 (Fig. 6*A*, *lanes 2–4*). Notably, siRNA-mediated knockdown of endogenous Cul3 caused a robust increase in the levels of Myc-RhoBTB1, and its levels were still reduced by

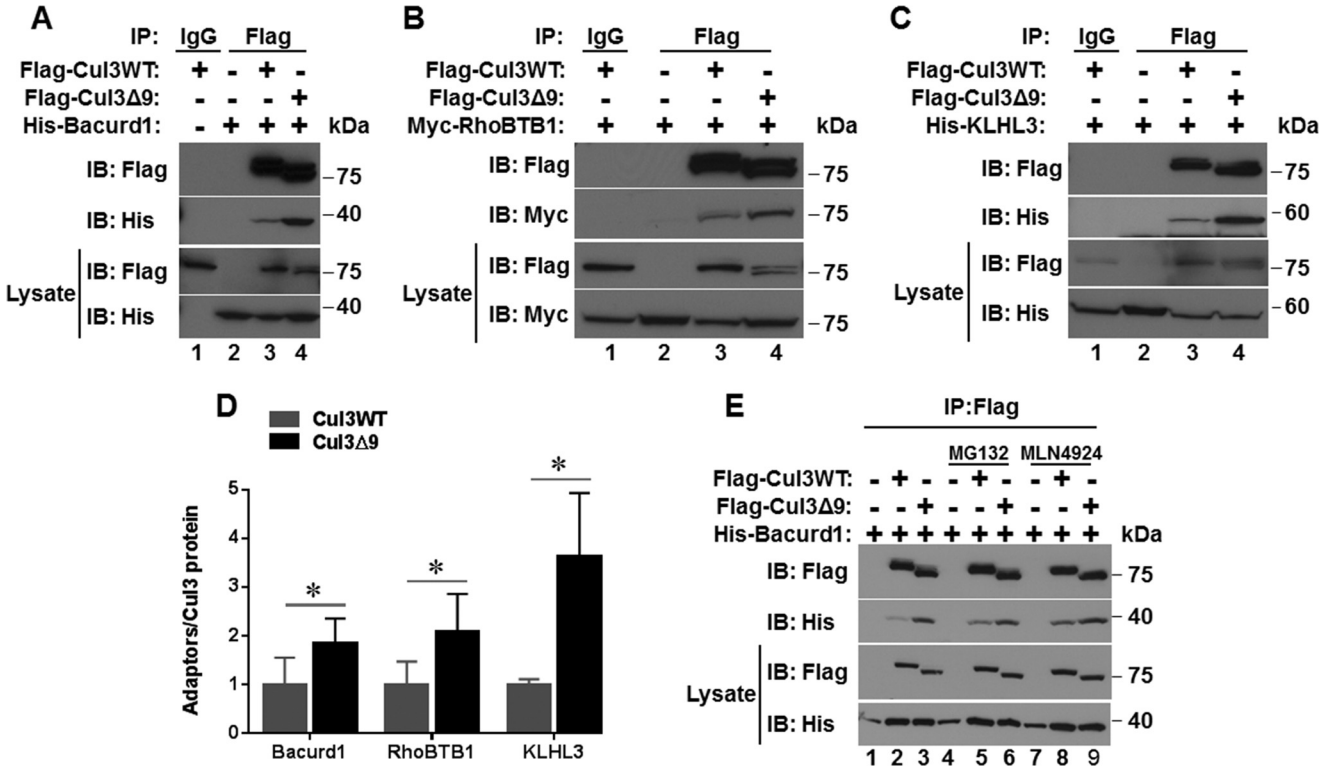

FIGURE 5. **Cul3Δ9 exhibits increased binding to adaptor proteins.** *A–C*, HEK293T cells were cotransfected with FLAG-Cul3WT or FLAG Cul3Δ9 along with His-Bacurd1 (*A*), Myc-RhoBTB1 (*B*), and His-KLHL3 (*C*). Cells were then lysed and subjected to immunoprecipitation with FLAG antibody. Western blotting was performed with whole cell lysates not subjected to IP as controls for antibody specificity. *IB*, immunoblot. *D*, quantification of immunoprecipitated adaptor proteins normalized to immunoprecipitated FLAG-Cul3. *Error bars* represent mean ± S.E. (n = 3, 5, and 3, respectively). *E*, immunoblot analysis of HEK293T^{Cul3KO} cells coexpressing FLAG-Cul3WT or FLAG-Cul3Δ9 with His-Bacurd1 and treated with 2 μM MG132 or 1 μM MLN4924 for 16 h. Immunoprecipitation of FLAG was performed, and precipitates were immunoblotted for His-Bacurd1. Representative blots of three experiments are shown. The position of each molecular weight size marker (in kilodaltons) is shown. *, $p < 0.05$ His-Bacurd1, Myc-RhoBTB1, or His-KLHL3 immunoprecipitated with FLAG-Cul3WT *versus* FLAG-Cul3Δ9.

coexpression with FLAG-Cul3WT but not by FLAG-Cul3Δ9 (Fig. 6, *A*, *lanes 6–8*, and *B*). The decrease in Myc-RhoBTB1 by HA-Cul3WT was blocked by MG132 or MLN4924 in HEK293T^{Cul3KO} cells (Fig. 6*C*). This suggests that RhoBTB1 is itself a substrate and that Cul3Δ9 has an impaired ability to degrade RhoBTB1. The effect of Cul3 expression was apparently substrate adaptor-dependent because there was no apparent effect on His-Bacurd1 expression (Fig. 6*D*). These observations suggest that Cul3Δ9 may act dominantly by increasing the expression levels of some substrate adaptors, which could skew the substrate adaptor composition of Cul3WT complexes and therefore alter substrate specificity. In contrast, substrate adaptors such as Bacurd1 that are not destabilized by Cul3 might also be sequestered from Cul3WT through their increased interaction with Cul3Δ9.

Cul3Δ9 Heterodimerizes with Cul3WT—It has been proposed that active forms of CRL complexes exist as homodimers that may be required for efficient ubiquitination of some substrates (14–16). Although the mechanism of dimer formation remains controversial, it is thought to occur through direct binding of substrate adaptors (16, 32) or through the Nedd8 interface (35). To test whether Cul3Δ9 exists in heterodimers with Cul3WT, we first determined whether Cul3WT and Cul3Δ9 can form a complex. We transfected HEK293T cells with two differently tagged Cul3 constructs and blocked proteasome and Cullin activity with MG132 and MLN4924, respectively, to prevent degradation of any potential heterodimeric complex. Using FLAG-agarose, we immunoprecipitated FLAG-Cul3WT or FLAG-Cul3Δ9 and probed for coprecipitation with Myc antibody to detect dimerization with Myc-tagged constructs (Fig. 7*A*). This analysis from cells treated with MG132 detected the presence of Cul3WT homodimers (Fig. 7*A*, *lane 1*), Cul3Δ9 homodimers (Fig. 7*A*, *lane 4*), and Cul3WT:Cul3Δ9 heterodimers (Fig. 7*A*, *lanes 2* and *3*). Similar complexes were observed in cells treated with MLN4924. However, when the analysis was performed without the inhibitors (Fig. 7*B*), pulling down FLAG-Cul3WT revealed the presence of homodimers with Myc-Cul3WT (Fig. 7*B*, *lane 2*) but did not detect heterodimerization with Myc-Cul3Δ9 (Fig. 7*B*, *lane 3*). Interestingly, pulling down FLAG-Cul3Δ9 revealed effective heterodimer formation with Myc-Cul3WT (Fig. 7*B*, *lane 4*). Therefore, more Cul3Δ9:Cul3WT heterodimer is detected when selecting for Cul3Δ9 complexes by IP than when selecting for Cul3WT complexes. This suggests that dimeric complexes containing Cul3Δ9 may be less stable than homodimers of Cul3WT. Homodimers of Cul3Δ9 also appeared unstable because they could only be detected from cells treated with MG132 or MLN4924. Importantly, identical results for each interaction were obtained in analyses performed by myc immunoprecipitation and FLAG immunoblotting (data not shown). Importantly, that the mutant Cul3Δ9 can form a heterodimer with Cul3WT, at least under certain conditions, suggests a

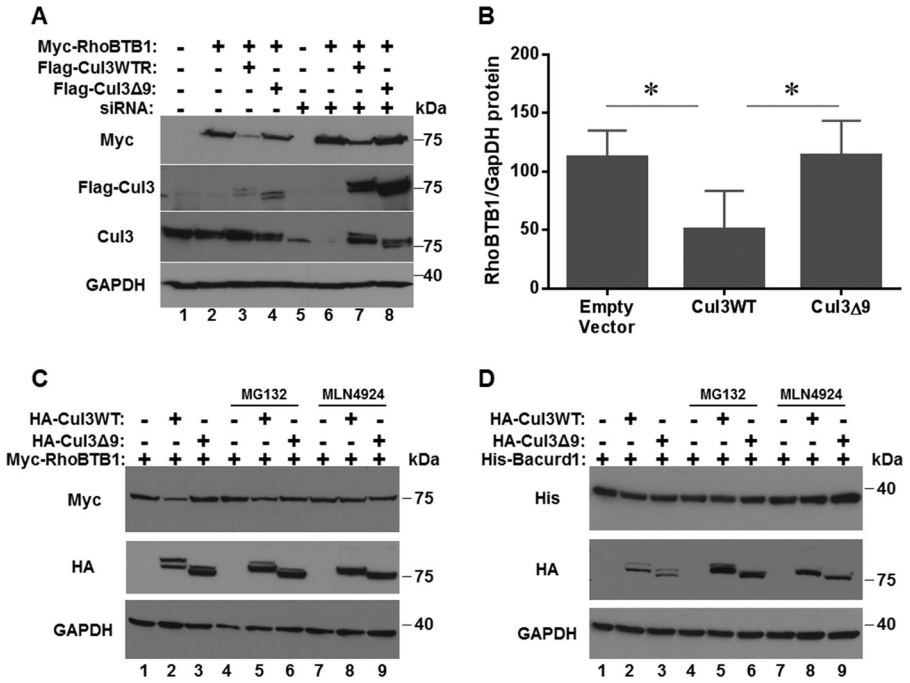

FIGURE 6. **Turnover of RhoBTB1 protein is impaired by Cul3Δ9.** *A*, immunoblotting assay showing RhoBTB1 protein levels in HEK293T cells transfected with Myc-RhoBTB1 alone or with FLAG-Cul3WT or FLAG-Cul3Δ9. Where indicated, cells were also transfected with endogenous Cul3 siRNA. Cells lysates were isolated 72 h post-transfection. *B*, quantification of Myc-RhoBTB1 protein normalized to GAPDH ($n = 3$). *, $p < 0.05$ Myc-RhoBTB1 with FLAG-Cul3WT *versus* Myc-RhoBTB1 alone or Myc-RhoBTB1 with FLAG-Cul3Δ9. *Error bars* represent mean ± S.E. *C* and *D*, immunoblot analysis of RhoBTB1 and Bacurd1 protein levels in HEK293T^{Cul3KO} cells coexpressing HA-Cul3WT or HA-Cul3Δ9 with Myc-RhoBTB1 (*C*) or His-Bacurd1 (*D*) and treated with 2 μM MG132, 1 μM MLN4924, or dimethyl sulfoxide. Representative blots of three experiments each for *C* and *D* are shown. The position of each molecular weight size marker (in kilodaltons) is shown.

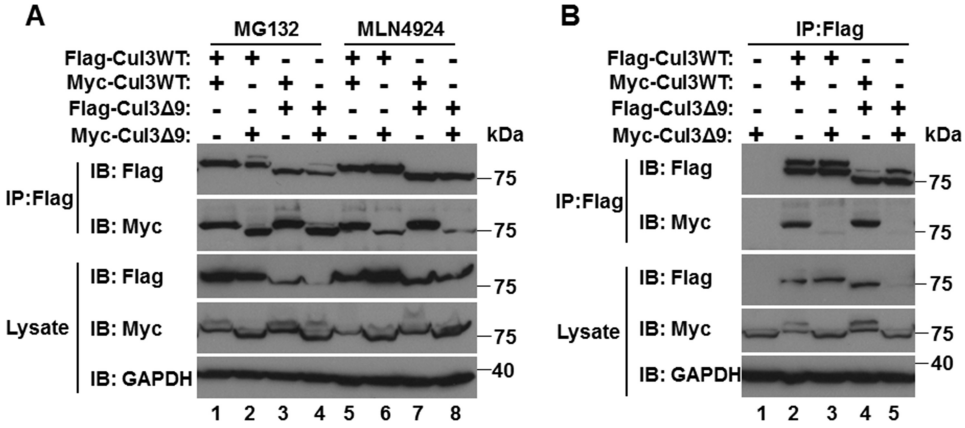

FIGURE 7. **Interaction between Cul3WT and Cul3Δ9.** Immunoblot (*IB*) analyses of dimer formation between Cul3WT and Cul3Δ9. HEK293T cells were transfected with two differently tagged Cul3Δ9; FLAG-Cul3Δ9 and Myc-Cul3Δ9 with either FLAG-Cul3WT or Myc-Cul3WT and His-Bacurd1. Transfected cells were also treated with 2 μM MG132 or 1 μM MLN4924 (*A*) or dimethyl sulfoxide (*B*) for 16 h. Using FLAG antibody, FLAG-Cul3WT or Cul3Δ9 was immunoprecipitated, and precipitates were probed with FLAG or Myc antibody to detect homodimer/heterodimer complexes. Representative blots of five experiments are shown. The position of each molecular weight size marker (in kilodaltons) is shown.

potential mechanism by which Cul3Δ9 may act dominantly to impair CRL3 complexes containing wild-type Cul3.

Active Cul3WT Is Reduced in the Presence of Cul3Δ9—The presence of unstable complexes containing Cul3Δ9 and Cul3WT suggests the possibility that expression of Cul3WT could be reduced by coexpression with Cul3Δ9. To test this, we assessed levels of the active (neddylated) form of Cul3WT in the presence of increasing amounts of Cul3Δ9. HEK293T^{Cul3KO} cells were transiently transfected with 1 μg of HA-Cul3WT alone or with increasing amount of HA-Cul3Δ9 (Fig. 8, *A* and *B*). The level of the neddylated Cul3WT was decreased significantly in the presence of increasing amounts of HA-Cul3Δ9 (Fig. 8*A*, compare *lane 2* to *lanes 3* and *4*). These data strongly suggest that Cul3WT is destroyed when part of unstable complexes with Cul3Δ9, causing a reduction in total active Cul3WT and reduced turnover of CRL3 targets such as RhoA.

Discussion

Hypertension is a key component of a metabolic syndrome that affects nearly one-third of the United States population. The prevalence of metabolic syndrome predisposes individuals to cardiovascular disease and increases their risk of adverse effects. Therefore, it is imperative that fundamental mechanisms that regulate blood pressure and cause hypertension be

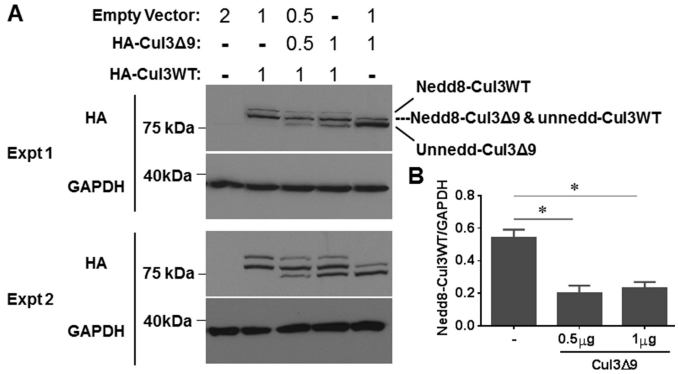

FIGURE 8. **Coexpression of Cul3Δ9 with Cul3WT decreases levels of neddylated Cul3WT.** Immunoblot analyses of protein levels of Cul3WT and Cul3Δ9. *A*, HEK293T^{Cul3KO} cells in a 6-well plate were cotransfected with HA-Cul3WT or HA-Cul3Δ9 or a combination of both, as indicated. Balanced amounts of empty vector were included to achieve an equal amount of total plasmid in each transfection. Cells lysates were isolated 48 h post-transfection. Blots of two (of three) independent experiments (*Expt*) are shown. *B*, quantification of neddylated Cul3WT protein normalized to GAPDH ($n = 3$). *, $p < 0.05$ Nedd-Cul3WT alone *versus* Nedd-Cul3WT with 0.5 or 1 μg of HA-Cul3Δ9. *Error bars* represent mean ± S.E. The position of each molecular weight size marker (in kilodaltons) is shown. The positions of neddylated (*Nedd8*) and un-neddylated (*unnedd*) Cul3 isoforms are indicated.

identified. Activation of RhoA and its downstream effector ROCK increases Ca^{2+} sensitization and contributes to agonist-induced vascular contraction (11–13). The involvement of RhoA/ROCK signaling in the development of cardiovascular diseases such as hypertension and atherosclerosis has attracted significant attention (36). We and others have previously reported a role for Cul3, an E3 ligase, in hypertension (4, 6). Cul3 regulates protein turnover by ubiquitination, thereby targeting them for proteasome-dependent degradation. Cul3 regulates vascular function and arterial pressure by controlling the turnover of RhoA and pharmacologically inhibiting the Cul3 pathway increases RhoA/ROCK signaling, leading to high blood pressure (4). In addition, blocking ROCK signaling in hypertension both in mouse models and humans significantly lowered blood pressure (11, 12, 37). Recently, dominant hypertension-causing mutations in Cul3 and KLHL3, a Cul3 adaptor protein, have been reported in PHAII patients (6). More than 90% of patients with dominant mutations in Cul3 that cause skipping of exon 9 (Cul3Δ9) have early-onset hypertension, and this has been attributed to an impaired Cul3 pathway in the renal system (6, 10). However, kidney-specific deletion of Cul3 in adult mice did not phenocopy PHAII patients because these mice exhibited unexpected physiological phenotypes, such as salt wasting and salt-sensitive hypotension, that are not observed in human patients with Cul3 mutations (23). These discrepancies suggest that either hypertension is not caused by a loss of Cul3 function specifically in the kidney or that Cul3Δ9 does not act only as a loss of function allele. In either case, the mechanisms by which Cul3Δ9 mutations impair extrarenal Cul3 function are likely contributors to hypertension. In this study, we used a model system consisting of genome-edited HEK293T cells to provide molecular insights into the concept that Cul3Δ9 has impaired ubiquitin ligase activity toward RhoA and may have dominance over wild-type Cul3.

This study provides evidence regarding why Cul3Δ9 exhibits impaired ubiquitin ligase activity. Cul3Δ9 association with Rbx1 is decreased, strongly suggesting that a reduced amount of E3 ligase is recruited to the CRL3 complex, thereby impairing transfer of ubiquitin from the E2 enzyme to the substrate. Ubiquitin ligase activity of the CRL3 complex also depends on the addition of a Nedd8 moiety to Lys-712 of Cul3 (24, 38). Neddylation of Cul3 is mediated by a complex comprising two E3 Nedd8 ligases, Rbx1 and DCN1, and an E2 Nedd8-conjugating enzyme, Ubc12, and also depends on the Nedd-8 activating enzyme (39). We show that neddylation of Cul3Δ9 is decreased significantly compared with Cul3WT. A recent report suggests that Cul3Δ9 is heavily neddylated (23), but we see no evidence of enhanced neddylation of Cul3Δ9. Because Rbx1 is also required for Cul3 neddylation, the reduced association of Cul3Δ9 with Rbx1 is consistent with its reduced neddylation and the reduced ubiquitin ligase activity of Cul3Δ9 complexes. Consistent with our findings, functional data from recent reports suggest that defective KLHL3/Cul3 leads to decreased ubiquitination and turnover of renal WNK1 and WNK4, therefore impairing the regulation of renal sodium and potassium (7–10).

We have also obtained data explaining the potential mechanisms of Cul3Δ9 dominance. First, Cul3Δ9 exhibits elevated binding to substrate adaptors compared with Cul3WT. This suggests that Cul3Δ9 may sequester adaptor proteins from Cul3WT complexes. Alternatively, Cul3Δ9 could influence total CRL3 activity by altering the availability of substrate adaptors that can associate with active complexes. For example, reduced turnover of RhoBTB1 in the presence of Cul3Δ9 may favor the formation of active CRL3 complexes targeting RhoBTB1-specific substrates rather than substrates that are specific to Bacurd1, such as RhoA. Finally, Cul3Δ9 may act dominantly by displacing Cul3WT from functional complexes. In this regard, members of the Cullin family (Cul1, Cul3, and Cul4) are known to exist in dimeric states required for the efficient transfer of ubiquitin to substrates (14, 35, 40–42). Therefore, incorporation of defective Cul3Δ9 into a complex with Cul3WT may interfere with Cul3WT function. A conjecture of the dominance effect because of Cul3WT:CulΔ9 heterodimer formation is that either the heterodimer is stable but inactive or that the heterodimer is inherently unstable. The latter possibility is supported by the significant stabilization of Cul3Δ9-containing dimers by inhibitors of the proteasome or CRL activity. Therefore, there are multiple mechanisms through which Cul3Δ9 may interfere with activities of coexpressed Cul3WT. One such mechanism consistent with our data is that the formation of a Cul3WT:Cul3Δ9 heterodimer results in turnover of the active form of Cul3WT, consequently reducing the activity of the coexpressed wild-type Cul3. It remains to be determined whether some or all of these are functional mediators of Cul3Δ9 dominance in reducing RhoA turnover by CRL3 complexes.

We recognize the potential limitation of using HEK293T cells to study the activities of Cul3 relevant to its potential role in vascular smooth muscle cells. However, our previous work has shown that reduced Cul3 expression in vascular smooth muscle cells also leads to increased RhoA expression (4). Therefore, disrupted CRL3 activity mediated by Cul3Δ9 is likely to have effects on RhoA expression in vascular smooth muscle similar to those shown in HEK293T cells. However, primary

smooth muscle cells are notoriously difficult to transfect and continuously change their phenotype in culture. HEK293T cells were attractive for this first study because they are easily transfected, which simplified the ablation of endogenous Cul3 by either siRNA or CRISPR-Cas9. Genome editing in parallel with overexpression of wild-type and mutant Cul3, Cul3 adaptors, and RhoA substrate provided a powerful experimental model to assess the fundamental mechanisms by which mutations in Cul3 disrupt RhoA turnover.

The data presented here indicate that Cul3 mutations present in PHAII patients impair ubiquitination of RhoA, a molecular switch that enhances Ca^{2+} sensitivity in smooth muscle contraction (43). Cul3 mutation impairs RhoA ubiquitination, thereby elevating RhoA protein and potentially increasing the RhoA pool that can be activated. RhoA/ROCK signaling is a major cellular regulator of agonist-induced smooth muscle contraction and, therefore, a potent contributor to hypertension. Clearly, studies are now needed to assess whether interference with wild-type Cul3 by Cul3Δ9 causes increased RhoA and Rho kinase activity in vascular smooth muscle and whether this translates to vascular dysfunction and hypertension. The generation of an inducible Cul3Δ9 transgene will facilitate these studies *in vivo*. It will also be important to determine whether the hypertension in PHAII patients occurs in greater part through defects in CRL3 complex formation and/or activity that impairs ubiquitination and turnover of RhoA. This study provides a mechanistic basis for supporting the concept that Cul3 complexes regulate the turnover of RhoA and can be used as a basis to determine whether this pathway is a regulator of vasomotor function and arterial blood pressure and how this regulation may be impaired by Cul3 mutations that cause hypertension.

Author Contributions—S. I. performed the experiments. L. A. generated the Cul3-edited HEK293T cells by CRISPR-Cas9 and performed data analysis. S. I., F. Q. and C. S. conceived and supervised the project, performed data analysis, and wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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