DATA SUPPLEMENT

A Novel *CUL3* Variant causing Familial Hyperkalemic Hypertension Impairs Regulation and Function of Ubiquitin Ligase activity

Harish E. Chatrathi[1,4], Jason C. Collins[2,4], Lynne A. Wolfe[1], Thomas C. Markello[1,3], David R. Adams[1,3], William A. Gahl[1,3], Achim Werner[2], Prashant Sharma[1*]

[1] NIH Undiagnosed Diseases Program, Common Fund, Office of the Director, National Institutes of Health, Bethesda, Maryland 20892, USA
[2] Stem Cell Biochemistry Unit, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892, USA
[3] Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland Bethesda, Maryland 20892, USA
[4] Share the first authorship position.

Short title: A novel *CUL3* variant in hypertension

***Correspondence**: Prashant Sharma, NIH Undiagnosed Diseases Program, National Human Genome Research Institute, 5625 Fishers Lane, Rockville, MD. Email: sharmap@mail.nih.gov

Supplemental Content:

METHODS

Whole exome and Sanger sequencing

Genomic DNA was extracted from the peripheral blood leukocytes using AutoGen FLEX STAR automated genomic DNA extraction and isolation system. Exome sequencing was performed by the NIH Intramural Sequencing Center on HiSeq 2000 Sequencing System (Illumina) using HumanOmniExpressExome-8v1-2_A SNP chip. Results were analyzed using GenomeStudio software (Illumina). Validation of the *CUL3* variant and segregation with disease were confirmed by Sanger sequencing. Genomic DNA flanking the site of deletion was amplified with HotStar Taq DNA polymerase (Qiagen, Valencia, CA) using the forward primer GCACAGAGCAACTGTTGGAAA and reverse primer AAACAACTGTCATCTGCTAAGTG.

PCR conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, extension at 72°C for 60 s, followed by a final extension step at 72°C for 10 min. PCR products were purified using ExoSAP-IT Express (Affymetrix) and cycle sequencing reactions were carried out using BigDye Terminator v3.1 mix (Applied Biosystems). The cycle conditions were initial denaturation at 96°C for 1 min followed by 25 cycles at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Excess dye terminators were removed using SAM/BigDye XTerminator bead solution mix (Applied Biosystems) and entire reaction was loaded on Applied Biosystems SeqStudio Genetic Analyzer instrument (Thermo Fisher). Sequence data were analyzed by Sequencher v5.0 software (Gene Codes Corporation, Ann Arbor, MI).

Cell culture, site-directed mutagenesis and transfection

Primary dermal fibroblasts from the patient were derived from a forearm skin punch biopsy. Unaffected pediatric primary dermal fibroblast cell lines (GM08398, GM01652, GM00942, GM00316) were obtained from the Coriell Institute of Medical Research (Camden, New Jersey). Fibroblasts and human embryonic kidney (HEK)293T cells (ATCC, Manassas, VA) were cultured using DMEM supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltman, MA), MEM nonessential amino acid solution (Sigma-Aldrich, St. Louis, MO), penicillin–streptomycin and 2 mM L-glutamine (Sigma-Aldrich). Cells were routinely tested for mycoplasma using the Universal Mycoplasma Detection Kit (ATCC). Proliferation of dermal fibroblasts was determined using Alamar blue dye. For each time point, 1000 cells were plated in four wells in a 96-well plate with complete DMEM media. 10% Alamar Blue (Thermo Fisher Sci.) was added at each day for 7 days, and a fluorescent reading was carried out 4 h later using SpectraMax microplate reader (Molecular Devices) with an excitation wavelength at 540 nm and an emission wavelength at 570 nm.

For transient transfection experiments in HEK293T cells, plasmids were obtained from Addgene (Watertown, MA) and GenScript USA (Piscataway, NJ). Patient specific deletion in CUL3 plasmid (pcDNA3-myc-CUL3Δ474-477) was generated using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and verified by DNA sequencing. Exponentially growing HEK293T cells were transiently transfected using Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cell culture media was replaced 24 h after transfection and cells were transfected for 48 h.

anti-FLAG immunoprecipitations

Immunoprecipitations (IP) were performed from extracts of HEK293T cells which were transiently transfected with FLAG-CUL3-WT and MYC-CUL3-Δ474-477 constructs and incubated for 24 hours at 37°C with 5% CO_2. Cells were collected in 1xPBS pelleted at 1000xg for 2 min and stored at -80°C. Pellets were resuspended in two pellet volumes of ice-cold lysis buffer [20 mM Hepes (pH 7.3) containing 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM EDTA, 0.1% NP-40, 1X protease inhibitors (Roche)] and passively lysed on ice for 30 min. Subsequently the lysate was briefly sonicated, clarified at 20,000xg for 30 min, and quantified using Pierce 660nm reagent. Lysates were incubated with pre-equilibrated anti-FLAG-M2 agarose beads (Sigma-Aldrich) for 1 hour at 4°C. Beads were washed three times with lysis buffer and bound proteins were eluted by addition of 2X urea sample buffer and analyzed by immunoblotting using indicated antibodies.

Purification of CUL3/RBX1 complexes for *in vitro* assays

CUL3/RBX1 WT and CUL3/RBX1 Δ474-477 complex proteins were purified as GST fusions from *E.coli* according to a previously established pGEX-based split-N-co-expression system.In brief, plasmids were transformed into Rossetta 2(DE3) cells and were grown in LB culture to a cell density of OD600 = 1.0-1.5, followed by 1mM IPTG induction overnight at 16°C for 16-20 h. Cells were harvested by centrifugation at 4000xg for 10 min and resuspended in lysis buffer [50 mM Tris, 500 mM NaCl, 2 mM EDTA, 2 mM DTT, pH 8.0, Protease Inhibitor Cocktail (Roche)]. Cells were lysed using a microfluidizer processor at 15000 psi and cell debris was removed by centrifugation at 50,000xg at 4°C for 30 min. Clarified lysates were incubated with pre-equilibrated Glutathione Sepharose 4B resin at 4°C for 2-4 hours. Beads containing GST-CUL3/RBX1 complexes were washed in 3-5 CV of wash buffer (50 mM Tris, 300 mM NaCl, and 1 mM DTT, pH 8.0). Subsequently, GST-CUL3/RBX1 complexes were eluted in wash buffer containing 10mM reduced glutathione. Eluted protein was dialyzed overnight at 4°C in separation buffer (100 mM Tris, 300 mM NaCl, and 1 mM DTT, pH 8.0). Complexes were concentrated using centrifuge concentrators and the GST-tag was cleaved by incubation at 30°C for 30 min with TEV protease. Cleaved protein was separated by HiLoad superdex 200pg size-exclusion chromatography and pure fractions were pooled, concentrated, and flash frozen in storage buffer (50 mM Tris, 200 mM NaCl, 1 mM DTT, 10% Glycerol, pH 8.0).

***In vitro* ubiquitination of CUL3/RBX1 complexes**

Ubiquitination of CUL3/RBX1 WT and CUL3/RBX1 Δ474-477 complexes was tested on non-neddylated forms. Ubiquitination reactions were performed in 20 µl reactions containing 1 µM CUL3/RBX1, 0.17 µM UBA1, 1.2 µM UBCH5, and 30 µM Ubiquitin in reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl2, pH 7.5). Reactions were initiated by addition of 5 mM ATP and incubated at 30°C for 1h. Ubiquitination of CUL3/RBX1 complexes was monitored at 0, 15, 30, 45, and 60 mins by quenching the reaction trough addition of urea sample buffer followed by SDS-PAGE and immunoblot analysis using anti-CUL3 antibodies. All Ubiquitination components were obtained from Boston Biochem. Ubiquitination was quantified using FIJI imaging software (ver. 1.52p). Level of Ubiquitination was calculated as a percentage of total unmodified protein at t=0 min followed by a baseline correction to 0 and plotted using GraphPad Prism 8 (ver. 8.4.1). Representative experiment of auto-ubiquitination of CUL3/RBX1-NEDD8 (n=4) are shown. Significant differences were determined by a multiple t-test of CUL3/RBX1 Δ474-477 vs CUL3/RBX1 WT.

***In vitro* neddylation and de-neddylation of CUL3/RBX1 complexes**

To test neddylation of CUL3/RBX1 WT and CUL3/RBX1 Δ474-477 complexes in vitro, 20 µl reactions containing 1 µM CUL3/RBX1, 0.5 µM APPBP1–UBA3, 0.9 µM UBE2M, and 3 µM NEDD8 were assembled in reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl2, pH 7.5). Reactions were initiated by addition of 5 mM ATP and incubated at 30°C for 2h. Neddylation of CUL3/RBX1 complexes was monitored at 0, 15, 30, 60, and 120 min by quenching the reaction through addition of urea sample buffer followed by SDS-PAGE and immunoblot analysis using anti-CUL3 antibodies. To test de-neddylation of CUL3 *in vitro*, CUL3/RBX1 WT and CUL3/RBX1 Δ474-477 complexes were neddylated for 2h as described above and reactions were depleted of ATP by addition of calf intestinal alkaline phosphatase and incubation at 37°C for 30

min. De-neddylation reactions were initiated by addition 0.6 µM human COP9 signalosome (Enzo Life sciences) and incubated at 30°C for 1h. Loss of NEDD8 was monitored by sampling the reaction at 0, 15, 30, 45, and 60 min by quenching the reaction through addition of urea sample buffer followed by SDS-PAGE and immunoblot analysis using anti-CUL3 antibodies. APPBP1–UBA3 was *in vitro* purified according to previously established protocols. All other neddylation components were obtained from Boston Biochem. Neddylation and de-neddylation blots were quantified using FIJI imaging software (ver. 1.52p). Gain and loss of NEDD8 was calculated as a ratio of CUL3/RBX1-NEDD8 / CUL3/RBX1 and plotted using GraphPad Prism 8 (ver. 8.4.1). Representative experiment of neddylation (n=5) and de-neddylation (n=6) were shown. Significant differences were determined by a multiple t-test of CUL3/RBX1 Δ474-477 vs CUL3/RBX1 WT.

Quantitative PCR

Total RNA was extracted from fibroblasts using the RNeasy Mini Kit (Qiagen). Contamination of residual genomic DNA from the RNA samples was removed by Turbo DNA free kit (Thermo Fisher Scientific) and single-stranded cDNA was synthesized using High Capacity RNA-to-cDNA kit (Applied Biosystems). Relative quantitation of *CUL3* transcripts was performed with an ABI7300 Genetic Analyzer (Applied Biosystems) using the FAM-labeled TaqMan Probe and primers mix (Hs00180183_m1; exon 14-15 *CUL3*) (Thermo Fisher Scientific) . Gene expression values were normalized to the expression of the reference gene RNA Polymerase II Subunit A (*POLR2A*) (Hs00172187_m1; Thermo Fisher Scientific).

Identification of CUL3 interacting proteins by mass spectrometry

Identification of CUL3-interacting proteins by mass spectrometry and compPASS analysis was performed as previously described. In brief, anti-FLAG immunoprecipitations for mass spectrometry analysis were performed from extracts of HEK293T cells transiently expressing FLAG-CUL3-WT or FLAG-CUL3-Δ474-477 (4 x 15 cm dishes per condition). Lysis was performed in two pellet volumes of lysis buffer (20 mM HEPES pH 7.3, 150 mM NaCl, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 5 mM EDTA, 5 mM EGTA, 0.2% NP-40) supplemented with 2 mM phenantroline and protease inhibitors (Roche) on ice followed by brief low-amplitude sonication and subsequent trituration through 25 gauge needles. Lysates were cleared by centrifugation, passaged through a 0.45 µm membrane filter, incubated with Protein G agarose for 30 mins at 4°C to remove nonspecific interactors, and incubated with ANTI-FLAG-M2 agarose (Sigma) for 1 h at 4 °C. After washing with lysis buffer, FLAG-tagged protein complexes were eluted with lysis buffer containing 0.5 mg/mL 3xFLAG peptide in three 15 min incubations at 30°C, 800rpm. Endogenous CUL3 immunoprecipitations for mass spectrometry were performed in a manner to preserve *in vivo* assemblies of CUL3 complexes following a protocol previously established for SCF complexes.[4] In brief, extracts form control or patient fiborblasts (2 x 15cm dishes) were lysed as described above but in the presence of ~100 fold excess of a sponge protein to suppress post-lysis, CAND1-mediated BTB adaptor exchange. Lysates were cleared by centrifugation, passaged through a 0.45 µm membrane filter, and incubated with mouse monoclonal anti-CUL3 antibodies crosslinked to Protein G agarose for 1h at 4°C. After washing with lysis buffer, endogenous CUL3 complexes were eluted with lysis buffer containing 0.5 mg/mL CUL3 peptide covering the epitope recognized by monoclonal anti-CUL3 antibodies in three 15 min incubations at 30°C, 800rpm.
Eluates from exogenous and endogenous CUL3 IPs were precipitated with TCA overnight, reduced, alkylated, separated from FLAG/CUL3 peptide via S-TrapTM mini columns (Protifi) and in-column digested with trypsin overnight. Tryptic digests were analyzed using an orbitrap Fusion

Lumos tribrid mass spectrometer interfaced to an UltiMate3000 RSLC nano HPLC system (Thermo Scientific) using data dependent acquisition. Initial protein identification was carried out using Proteome Discoverer (V2.4) software (Thermo Scientific). Search results from Proteome Discoverer were incorporated into Scaffold4 for relative quantification using spectral counting. For each immunoprecipitation condition, 2-3 biological replicates were performed that were analyzed in duplicate by mass spectrometry (2 technical replicates per biological replicate). To compare CUL3 WT and Δ474-474, total spectral counts for each interactor were first normalized to 1000 TSC CUL3 for ectopically expressed CUL3 and 200 TSC for endogenous CUL3 and then expressed relative to WT/controls in fold-change (log_2) and plotted as heatmap using GraphPad.

Clinical Summary

The patient is a 3-year-old Caucasian male of non-consanguineous European Caucasian descent. His parents and siblings had no known medical problems. The proband presented to the NIH with global developmental delay, hyperkalemia, hypertension, gastroesophageal reflux disease, chronic aspiration, frequent pulmonary infections and dysmorphic facial features with triangular face and chin cleft. He was born via vaginal delivery at 37.5 weeks gestation to a 33 year old, G8P3 mother with a history of 4 miscarriages due to blood clotting. During the pregnancy, maternal exposures included lovenox, heparin, and aspirin due to a history of deep vein thrombosis, pulmonary embolism, and miscarriages. His mother was prescribed antibiotics during pregnancy due to pneumonia, bronchitis, and sinusitis. A fetal stress test performed at 16 weeks of gestation showed a heart rate under 100 beats per minute (normal 109-110). Intrauterine growth deficiency persisted throughout gestation along with concerns of placental insufficiency at 30 weeks' gestation. Maternal development of shingles at 36 weeks' gestation was treated with a single dose of valacyclovir and oxycodone/acetaminophen. Birth weight was 2.30 kg and length 43.2 cm. The left testis was undescended. The infant was cyanotic for 12-24 hours immediately post-natally with pulse oximetry readings as low as 68%. He was eventually discharged with diagnoses of gastroesophageal disease and bronchospasms after a total of 7 days in the neonatal intensive care unit.

The patient was diagnosed with failure to thrive during the first year of life, with both height and weight significantly below the fifth percentile by 5 months of age. At 8 months of age, endocrinology evaluation revealed multiple undetectable IGF-1 levels. Growth hormone levels during the first 19 months of life were elevated with an average value of 13.5 ng/ml. He began growing at a rate of 5.4 cm/year, reaching a height at the 13^{th} percentile after starting growth hormone therapy. He suffered from multiple pulmonary infections and was treated with oral glucocorticoids approximately five times during his first year. Chest x-rays showed atelectasis of the both lower lobes of the lung during the first twelve months of life. Serum chemistries showed hyperkalemia and metabolic acidosis in infancy during hospitalizations. The patient was treated with chlorothiazide and bicarbonate which improved the patient's electrolyte imbalances. However, the patient did not have an official diagnosis at this time. An examination at 19 months revealed hypotonia, hyporeflexia and facial asymmetry. MRI of the brain showed nonspecific thinning and volume loss in the white matter lining of the lateral ventricular atrium. The patient's mother stated that he suffered from occasional seizures during his development. At 3 years of age, the patient had nonspecific dysmorphic facial features including triangular facies, in addition to short stature and thin habitus. He exhibited speech dyspraxia, could not follow verbal

commands, and displayed increased drooling with performance of fine motor skills. He also showed diffuse hypermobility in joints, including the elbows and wrists.

Blood pressures were consistently elevated (> 110 mmHg systolic) when taken off diuretics during his inpatient admission to the UDP, with a mean systolic blood pressure of 125 mmHg over the course of his evaluation. He had persistent hyperkalemia with serum potassium levels between 5 and 6.7 mmol/L while his serum aldosterone levels were within normal limits. He was acidotic with a normal non-anion gap and showed mild hyperchloremia with ultrasound evidence of nephrolithiasis. As a result, he was prescribed sodium citrate. His short stature was determined not to be the result of growth hormone deficiency due to his normal IGF-1 levels during clinical presentation at the UDP. The patient was officially diagnosed with PHAII at age 9, after discovery of a heterozygous variant in *CUL3*.

Supplemental Figures

Figure S1

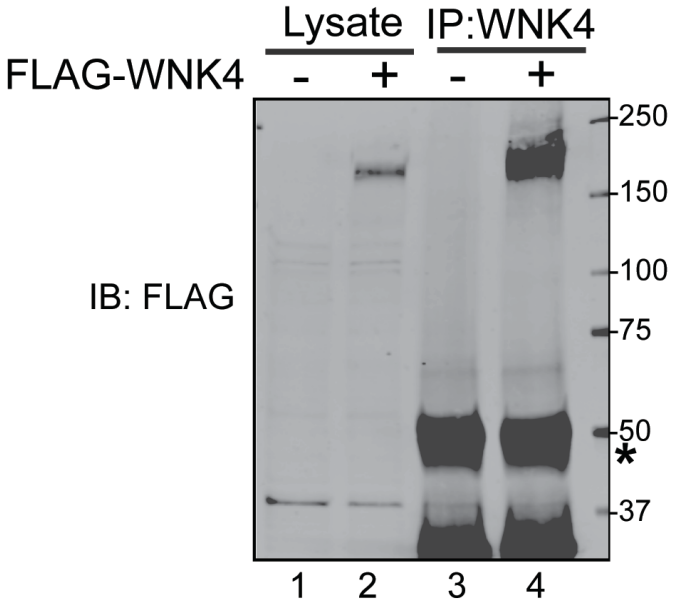

Figure S1. Specificity of WNK4 antibody.

HEK293T cells were transiently transfected with an empty vector or FLAG-WNK4 expression construct. Protein extracts were immunoprecipitated with an anti-WNK4 antibody that was used for the immunoprecipitation and detection of the endogenous WNK4 in fibroblasts lysates. Immunoblotting of WNK4 immunoprecipitates with anti-FLAG antibody detects FLAG-WNK4, migrating as a single band between 150 and 250 kDa molecular weight markers (lanes 2 and 4).* shows heavy and light chains of the antibody.

Figure S2

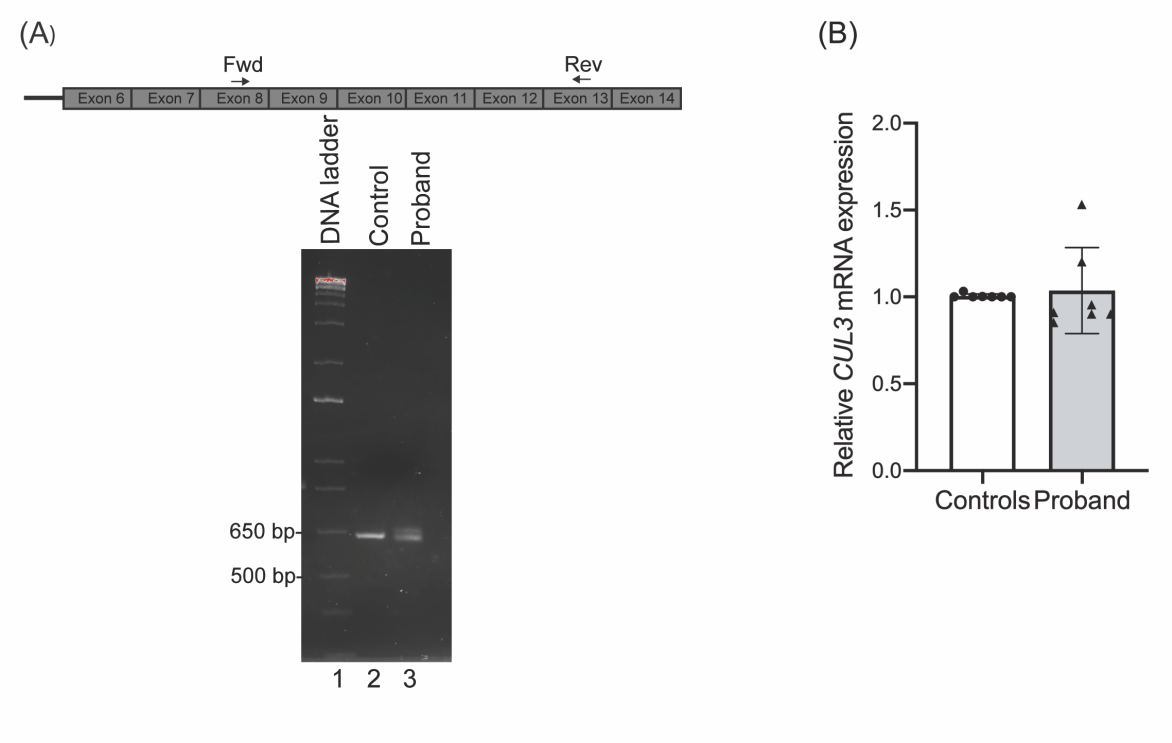

Figure S2: *CUL3* variant does not lead to exon 9 deletion and affects mRNA expression.

(A) An agarose gel image of the PCR products shows no deletion of exon 9 in cDNA from the patient. The upper schematic depicts the position of forward and reverse primers used in the PCR assay. Lane 1 is a 1Kb molecular weight DNA marker. Lane 3 shows the presence of both normal and truncated (in-frame deletion of 12 nucleotides) transcripts in the patient.
(B) Real-time quantitative PCR (qPCR) analysis shows no statistically significant difference in *CUL3* mRNA expressions between two unaffected controls and patient fibroblasts (error bars indicate ± SD). Taqman probes used in the assay anneal to the junction between exon14 and 15 (after the variant) in *CUL3*. *POLR2A* was used as a housekeeping gene to normalize the expression of *CUL3*.

Figure S3

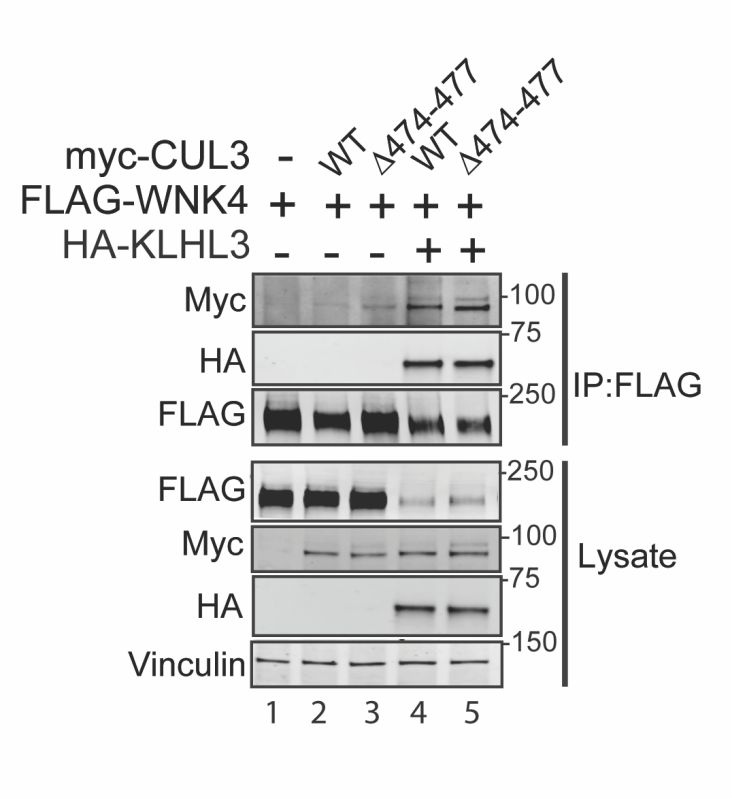

Figure S3: KLHL3 mediates the interaction between CUL3 and WNK4

HEK293T cells were co-transfected with WT-myc-CUL3 or myc-CUL3Δ474-477 along with FLAG-WNK4 in the absence or presence of HA-KLHL3. Cells lysates were subjected to immunoprecipitation with an anti-FLAG antibody. Immunoblotting of precipitates with anti-Myc and anti-HA antibodies revealed interactions of WNK4 with both WT-CUL3 and CUL3Δ474-477 (lanes 2 and 3). The interaction was markedly higher in the presence of ectopically expressed KLHL3 (lanes 4 and 5). Direct immunoblotting of lysates with anti-FLAG showed that the addition of KLHL3 reduced the available pool of FLAG-WNK4, likely due to CUL3 mediated ubiquitination. Immunoblotting of lysates with anti-Vinculin antibody confirmed the equal loading of samples.

Figure S4

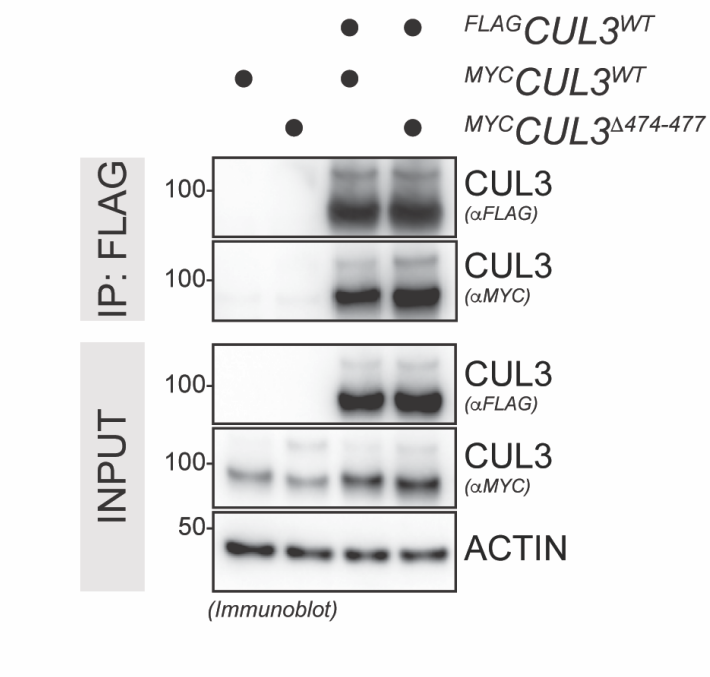

Figure S4: CUL3WT forms heterodimer with CUL3Δ474-477.

To determine if CUL3Δ474-477 could still form heterodimers with CUL3-WT, HEK293T cells were co-transfected with FLAG-CUL3-WT and either MYC-CUL3-WT or MYC-CUL3Δ474-477. Protein extracts were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-FLAG and anti-Myc antibodies to detect interactions between CUL3-WT and CUL3Δ474-477. Immunoblots indicated that the aa474-477 deletion does not disrupt CUL3's ability to heterodimerize with CUL-WT. Immunoblotting of lysates with anti-Actin antibody confirmed the equal loading of samples.