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# Dual Gain and Loss of Cullin 3 Function Mediates Familial Hyperkalemic Hypertension

Ryan J. Cornelius

*Oregon Health and Science University*

Chang Zhang

*Shanghai Jiao Tong University School of Medicine*

Kayla J. Erspamer

*Oregon Health and Science University*

Larry N. Agbor

*Carver College of Medicine, University of Iowa*

Curt D. Sigmund

*Carver College of Medicine, University of Iowa*

*See next page for additional authors*

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**Authors**

Ryan J. Cornelius, Chang Zhang, Kayla J. Erspamer, Larry N. Agbor, Curt D. Sigmund, Jeffrey Singer, Chao-Ling Yang, and David H. Ellison

# Dual gain and loss of cullin 3 function mediates familial hyperkalemic hypertension

Ryan J. Cornelius<sup>1</sup>, Chong Zhang<sup>2</sup>, Kayla J. Erspamer<sup>1</sup>, Larry N. Agbor<sup>3</sup>, Curt D. Sigmund<sup>3</sup>, Jeffrey D. Singer<sup>4</sup>,  
Chao-Ling Yang<sup>1,\*</sup>, and David H. Ellison<sup>1,5,\*</sup>

1. Division of Nephrology and Hypertension, Department of Medicine, Oregon Health and Science University, Portland, OR, USA
2. Department of Nephrology, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China
3. Department of Pharmacology, UIHC for Hypertension Research, Carver College of Medicine, University of Iowa, Iowa City, IA, USA
4. Department of Biology, Portland State University, Portland, OR, USA
5. VA Portland Health Care System, Portland, OR, USA

\*Chao-Ling Yang and David H. Ellison contributed equally to this work.

Running Foot: Cul3 $\Delta$ 403-459 degrades KLHL3 via dual pathways

David H. Ellison, M.D.

Division of Nephrology and Hypertension, SON440

Oregon Health and Science University

3181 SW Sam Jackson Park Road

Portland, OR 97239

Phone: 503 494-4465

Email: ellisond@ohsu.edu

31 **Abbreviations:**  
32 Cul3, Cullin 3; FHHT, familial hyperkalemic hypertension; CSN, COP9 signalosome; WNK, with-no-lysine  
33 kinase; NCC, Na-Cl cotransporter; DCT, distal convoluted tubule; SPAK, serine/threonine protein kinase 39;  
34 OSR1, oxidative stress-response 1; CRL, cullin-RING ligase; KLHL3, kelch-like 3; NEDD8, neuronal  
35 precursor cell expressed developmentally down-regulated protein 8; JAB1, jun activation domain-binding  
36 protein-1; 4HB, 4-helix bundle; WT, wild type; BTB, bric-a-brac, tramtrack, broad-complex; CAND1, cullin-  
37 associated and NEDD8-dissociated protein 1; Keap1, kelch-like ECH-associated protein-1; Nrf2, nuclear factor  
38 erythroid 2-related factor 2; 3-MA, 3-methyladenine.

39

40 **Abstract**

41       Familial hyperkalemic hypertension is caused by mutations in WNK kinases, or in proteins that mediate  
42 their degradation, KLHL3 and cullin 3 (Cul3). While the mechanisms by which WNK and KLHL3 mutations  
43 cause the disease are now clear, the effects of the disease-causing Cul3 $\Delta$ 403-459 mutation remain controversial.  
44 Possible mechanisms including hyperneddylation, altered ubiquitin ligase activity, decreased association with  
45 the COP9 signalosome (CSN), and increased association with and degradation of KLHL3 have all been  
46 postulated. Here, we systematically evaluated the effects of Cul3 $\Delta$ 403-459 using cultured kidney cells. We first  
47 identified that the catalytically active CSN subunit JAB1 does not associate with the deleted Cul3 4HB domain,  
48 but instead with the adjacent  $\alpha/\beta_1$  domain, suggesting that altered protein folding underlie the impaired binding.  
49 Inhibition of deneddylation, with JAB1 siRNA, increased Cul3 neddylation, and decreased KLHL3 abundance,  
50 similar to the Cul3 mutant. We next determined that KLHL3 degradation has both ubiquitin ligase-dependent  
51 and -independent components. Proteasomal KLHL3 degradation was enhanced by Cul3 $\Delta$ 403-459; however,  
52 autophagic degradation was also upregulated by this Cul3 mutant. Finally, to evaluate whether deficient  
53 substrate adaptor was responsible for the disease, we restored KLHL3 to WT-Cul3 levels. In the absence of  
54 WT-Cul3, WNK4 was not degraded, demonstrating that Cul3 $\Delta$ 403-459 itself cannot degrade WNK4;  
55 conversely, when WT-Cul3 was present, as in diseased humans, WNK4 degradation was restored. In  
56 conclusion, deletion of exon 9 from Cul3 generates a protein that is itself ubiquitin ligase-defective, but also  
57 capable of enhanced autophagocytic KLHL3 degradation, thereby exerting dominant-negative effects on the  
58 WT-allele.

59

60 **Keywords:** Cullin-RING ubiquitin ligase, Neddylation, Deneddylation, JAB1

61

62

63

## 64 Introduction

65 With-no-lysine (WNK) kinases control blood pressure and potassium homeostasis, predominantly by  
66 regulating membrane expression and activity of the thiazide-sensitive Na-Cl cotransporter (NCC) in the distal  
67 convoluted tubule (DCT). These kinases signal via serine/threonine protein kinase 39 (SPAK; *STK39*) and  
68 oxidative stress-response 1 (OxSR1 or OSR1; *OXSRI*), which directly phosphorylate and activate the transport  
69 protein (19, 30). The human Mendelian disease Familial Hyperkalemic Hypertension (FHHT, also called  
70 pseudohypoaldosteronism type 2 or Gordon syndrome) results from activation of this signaling pathway in the  
71 distal nephron (21). FHHT patients exhibit hyperkalemia, metabolic acidosis, and hypertension, symptoms that  
72 largely disappear during treatment with thiazide diuretics (13). FHHT can be caused by mutations in WNK1 or  
73 WNK4 (32), or in the cullin-RING ligase (CRL) proteins cullin 3 (*Cul3*) (4) and kelch-like 3 (*KLHL3*) (28).  
74 *Cul3* is part of an E3 ubiquitin ligase complex that regulates protein degradation. CRLs do not degrade proteins  
75 directly, but instead attach strings of ubiquitin moieties to a protein, thereby targeting it for degradation,  
76 typically within the proteasome. Cullin acts as a scaffold protein for the other CRL subunits. It is now clear that  
77 WNK kinases are targets for CRLs. The substrate adaptor KLHL3, binds both WNK kinases and *Cul3*, bringing  
78 the WNK into proximity to the catalytic region of the CRL, thereby permitting ubiquitylation (18, 25). The  
79 WNK4 or KLHL3 mutations that cause disease, do so by disrupting these binding reactions, permitting WNKs  
80 to accumulate (31). The *Cul3* mutations that cause FHHT, however, do not decrease substrate adaptor binding  
81 (27, 31); although mouse models indicate that WNKs are not degraded normally (24), the precise mechanisms  
82 involved remain controversial.

83 An important feature of CRL activity is the attachment of NEDD8 (neuronal precursor cell expressed  
84 developmentally down-regulated protein 8) through a process called neddylation. NEDD8 attachment is  
85 required to activate CRLs by increasing the flexibility of the cullin-ring structure, allowing the transfer of  
86 ubiquitin from the RING protein to the target substrate (3, 20, 23). The reverse process, deneddylation, is  
87 facilitated by the multi-subunit COP9 signalosome (CSN) complex. The CSN interacts with CRLs and removes  
88 NEDD8 through its catalytically-active CSN5 subunit, also known as JAB1 (jun activation domain-binding  
89 protein-1) (6). Although a simple model originally suggested that neddylated *Cul3* is active, whereas  
90 unneddyated *Cul3* is inactive, inhibition of CSN paradoxically increases, rather than decreases, the abundance  
91 of substrate proteins *in vivo* (22). It appears instead that, while neddylation of cullins is indeed essential to  
92 activate them, it also makes them unstable and prone to degradation (35). Thus, the effects of neddylation on  
93 cullin activity and abundance are complex.

94 All known FHHT-causing *Cul3* mutations cause deletion of exon 9, resulting in a mutant protein that  
95 lacks 57 amino acid residues (*Cul3* $\Delta$ 403-459) (4). Previous work by our group (14), and confirmed by others  
96 (24), showed that *Cul3* $\Delta$ 403-459, expressed in cells, is hyperneddylated, compared with wild type (WT),  
97 suggesting either that it is more susceptible to neddylation or that it is resistant to deneddylation. Schumacher *et*  
98 *al.* (24) confirmed that *Cul3* $\Delta$ 403-459 had impaired deneddylation and compromised CSN binding, suggesting

99 that the deleted segment was responsible. Exon 9 of Cul3 encodes the 4-helix bundle (4HB) domain. Min et al.  
100 (15) demonstrated that the CSN binding site for the closely related cullin 1 protein was located within the 4HB  
101 and  $\alpha/\beta_1$  domains. However, the CSN binding site for Cul3 has yet to be determined.

102 Cul3 $\Delta$ 403-459 has an increased association with bric-a-brac, tramtrack, broad-complex (BTB)-Kelch  
103 substrate adaptor proteins, including KLHL3 (10, 14, 24). The FHHt Cul3 mutant strongly ubiquitylated  
104 KLHL3 (14, 24) leading to decreased abundance, *in vitro* (14). However, administration of the non-specific  
105 neddylation inhibitor, MLN4924, which prevents NEDD8 conjugation and presumably inactivates CRLs, only  
106 partially normalized KLHL3 abundance. Additionally, the enhanced interaction of KLHL3 with Cul3 $\Delta$ 403-459  
107 remained in the absence of neddylation. The results indicate that Cul3 $\Delta$ 403-459 may have neddylation-  
108 dependent and neddylation-independent effects. Here, we examined the mechanisms and consequences of  
109 Cul3 $\Delta$ 403-459 hyperneddylation, and identified novel ligase dependent, and ligase independent mechanisms for  
110 the human disease.

## 112 **Materials and Methods**

### 113 *Antibodies*

114 Antibodies used are described in Table 1.

### 116 *Cell culture, plasmids, and transfections*

117 For cell culture experiments HEK293 cells were used unless otherwise stated. CRISPR-Cas9-edited  
118 Cul3 knockdown HEK293T cells (HEK293T<sup>Cul3-KO</sup>) were previously reported (10). Cells were maintained in  
119 DMEM supplemented with 10% FBS, 25 mM HEPES, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin. Cells  
120 were transiently transfected using Lipofectamine 2000 (Ambion, Foster City, CA, USA; Invitrogen). Cul3  
121 constructs were made by amplifying FLAG-Cul3 WT DNA using Phusion Hot Start II DNA Polymerase  
122 (Thermo Fisher Scientific, Boston, MA, USA) with the appropriate primers, purified with the PureLink PCR  
123 Purification Kit (Invitrogen) and properly digested. The products were then extracted using the UltraClean  
124 GelSpin DNA Extraction Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) and ligated into N-terminal GST  
125 tag mammalian plasmid, pSF-CMV-Puro-NH2-GST (Oxford Genetics, Oxford, UK), with T4 DNA Ligase  
126 (New England BioLabs, Ipswich, MA, USA). Ligated constructs were transformed using DH5 $\alpha$  competent cells  
127 (Thermo Fisher Scientific) and plasmid DNA was purified with either the QIAprep Spin Miniprep Kit or  
128 HiSpeed Plasmid Midi Kit (Qiagen, Hilden, Germany). Sanger sequencing was performed for all constructs.

129 For siRNA experiments, either 40 nM of COPS5 siRNA (Ambion) or control siRNA was transfected  
130 along with DNA plasmids. Cells were harvested at 36 h post-transfection.

131 For cycloheximide chase experiments, cycloheximide was added 36 h after transfection at a  
132 concentration of 100  $\mu$ g/ml and the cells were lysed at the time points indicated.

133 For MG132, chloroquine, 3-methyadenine, MLN4924, and tBHQ experiments, drug was added to cells  
134 18 h before harvesting at the concentrations given.

135 For ubiquitin assay experiments, cells were co-transfected with HA-tagged ubiquitin DNA plasmid.  
136 Cells were lysed 48 h after transfection in cell lysis buffer containing 10 mM N-ethylmaleimide.  
137 Immunoprecipitation and Western blotting was carried out as below.

### 138 *Immunoprecipitation and Western blotting*

139 Transfected cells were harvested in 0.5% Triton X-100 in PBS cell lysis buffer containing enzyme  
140 inhibitors. For immunoprecipitation cell lysate was pre-cleared with protein A-sepharose beads for 1-2 hours.  
141 Cell lysate was then incubated with Glutathione Sepharose 4B medium (GE Healthcare, Piscataway, NJ, USA)  
142 for 2 h at room temperature, primary antibody and Protein A-Sepharose 4B medium (GE Healthcare) or anti-  
143 FLAG Affinity Gel (Biotool, Houston, TX, USA) overnight at 4° C. Protein samples were separated by  
144 electrophoresis on 4-12% NuPAGE bis-tris polyacrylamide gels (Thermo Fisher Scientific) or 4-15% Criterion  
145 TGX stain-free gels (Bio-Rad Laboratories, Hercules, California, USA) and transferred to Immobilon-P PVDF  
146 membranes (EMD Millipore, Billerica, MA, USA). For all experimental conditions performed in triplicate, each  
147 well represents a unique transfection. Stain-free imaging was used as a total protein loading control, unless  
148 otherwise stated. Membranes were blocked with 5% milk in PBS for 1 h at room temperature before incubation  
149 with primary antibody in blocking buffer for 1 h at room temperature or overnight at 4° C. Appropriate HRP-  
150 conjugated secondary antibody in blocking buffer was added to membranes for 1 h at room temperature.  
151 Membranes were developed using enhanced chemiluminescence, Western Lightning Plus-ECL (Perkin Elmer,  
152 Waltham, MA, USA), and proteins were visualized using PXi digital imaging system (Syngene, Frederick, MA,  
153 USA).

### 154 *Statistics*

155 Data are presented as individual values as well as mean  $\pm$  SEM. Differences between two groups were  
156 determined using two-tailed unpaired Student's t-test and differences between multiple groups were analyzed  
157 using one-way ANOVA followed by Tukey's multiple comparisons test. A *P* value of less than 0.05 was  
158 considered significant. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad  
159 Software, San Diego, CA, USA).

## 160 **Results**

### 161 *CSN binds to Cul3 at the $\alpha/\beta_1$ domain.*

162 CRLs are activated by NEDD8 attachment, but for full functionality, they must also be deneddylated.  
163 The deneddylation of Cul3 appears to be disrupted in Cul3 $\Delta$ 403-459 FHHt, as the mutant protein is more highly  
164 neddylated than WT, at least when expressed in cultured cells (14, 24). Schumacher et al. reported that  
165



168 Cul3 $\Delta$ 403-459 exhibits decreased interaction with JAB1 (24), suggesting that the deleted domain may include  
169 the CSN binding site. The crystal structure of CSN with the highly homologous cullin 1 (12) and cullin 4A (5),  
170 showed that the CSN2 subunit interacts directly with the C-terminal domain. Additionally, Min et al. (15)  
171 determined that the CSN binding site for cullin 1 (which is structurally similar to Cul3) was located specifically  
172 within the C-terminal domains 4HB and  $\alpha/\beta_1$ . Inspection of the Cul3 gene revealed that exon 9 (deleted in  
173 FHHt-causing Cul3 mutations) encodes the 4HB domain (see Figure 1A). To determine sites of CSN interaction  
174 with Cul3, we generated Cul3 deletion constructs (Figure 1A). We first confirmed that, compared with WT-  
175 Cul3, there was minimal JAB1 precipitation by Cul3 $\Delta$ 403-459 (Figure 1B). Similarly, the Cul3 construct  
176 containing N-terminal residues 1-402, which lacks the 4HB domain and the adjacent  $\alpha/\beta_1$  domain showed  
177 nominal precipitation of JAB1 (Figure 1C). Surprisingly, inclusion of the 4HB domain with the N-terminal  
178 region also showed low binding to JAB1 (Figure 1C, Cul3 1-459). These results indicate clearly that Cul3  
179 amino acids 403-459 (containing the 4HB domain) are not sufficient for binding to the CSN. This suggests that  
180 the Cul3 $\Delta$ 403-459 mutation does not impair Cul3-CSN binding directly, but rather disrupts protein folding  
181 within a site C-terminal to the 4HB domain; this suggestion is consistent with structural modeling of wild type  
182 and mutant Cul3 (24).

183 To identify the specific binding site for the CSN and confirm that the 4HB does not bind JAB1 we  
184 developed individual Cul3 domain constructs for 4HB and  $\alpha/\beta_1$ . Additionally, we generated a construct  
185 containing both the 4HB and  $\alpha/\beta_1$  domains (4HB: $\alpha/\beta_1$ ), an N-terminal construct containing cullin repeat  
186 sequences (R1:R2:R3) and a C-terminal construct containing domains WH-A,  $\alpha/\beta_2$ , and WH-B (WH-  
187 A: $\alpha/\beta_2$ :WH-B). JAB1 immunoprecipitated with the Cul3 construct containing both the 4HB and  $\alpha/\beta_1$  domains  
188 and with  $\alpha/\beta_1$  alone, but not with 4HB alone (Figure 1D). JAB1 was not precipitated with Cul3 constructs that  
189 lacked the  $\alpha/\beta_1$  domain, R1:R2:R3 and WH-A: $\alpha/\beta_2$ :WH-B. The results indicate that the first  $\alpha/\beta$  domain of Cul3  
190 is the binding site for JAB1 and therefore the CSN.

191 To verify that the  $\alpha/\beta_1$  domain is the binding site for the CSN we developed a full-length Cul3 construct  
192 in which the  $\alpha/\beta_1$  was deleted (Cul3 $\Delta$ 461-586). JAB1 was not precipitated with Cul3 $\Delta$ 461-586 (Figure 1E). As  
193 expected, Cul3 $\Delta$ 461-586 also showed enhanced neddylation compared to WT-Cul3. These results further  
194 confirm that the CSN binding site is contained within the  $\alpha/\beta_1$  domain of Cul3.

### 195 *CSN inhibition enhances Cul3 neddylation and reduces KLHL3 and WNK4 abundance.*

196 Neddylation of cullins has paradoxical effects, with both neddylation and deneddylation being required  
197 for normal CRL function. Because Cul3 $\Delta$ 403-459 exhibits decreased interaction with JAB1 (24), we examined  
198 the effects of inhibiting JAB1 activity on Cul3 in HEK293 cells. Cells were transfected with siRNA to reduce  
199 endogenous JAB1. Western blotting for endogenous Cul3 exhibited a more neddylated Cul3 (as detected by an  
200 increase in the higher molecular weight Cul3 band) when JAB1 was reduced, compared to control (Figure 2).  
201 Similarly, probing the blot with an antibody against NEDD8 showed a greater abundance of neddylated Cul3  
202

when JAB1 was knocked down, as the antibody recognized a product at the molecular weight of Cul3. The effects of JAB1 inhibition on the protein abundance of the Cul3 substrate adaptor KLHL3 and substrate WNK4 were also determined. Abundance of overexpressed KLHL3 and WNK4 were lower, in cells transfected with JAB1 siRNA, compared to control siRNA, indicating that JAB1 knockdown and increased neddylation activates Cul3 in transfected cells.

#### *Cul3 $\Delta$ 403-459 causes decreased stability of KLHL3*

We showed previously that KLHL3 protein abundance was lower when co-expressed with Cul3 $\Delta$ 403-459 than with WT Cul3 in HEK293 cells (14), suggesting that degradation was more rapid in the presence of mutant Cul3. To test this, we measured the stability of KLHL3 using the cycloheximide chase assay. KLHL3 abundance was significantly lower 24 hours after cycloheximide treatment when co-transfected with Cul3 $\Delta$ 403-459 compared to WT Cul3, but the apparent degradation rates at other time points were not different (Figure 3A). As KLHL3 co-transfected with WT Cul3 was stable at least 24 h after cycloheximide treatment (the long stability of KLHL3 has been previously published (17)), which can lead to anomalous results in cycloheximide chase experiments (37), we decided to examine a canonical CRL substrate, WNK4, using a similar approach. Similar to the Cul3-KLHL3 experiments, WNK4 was stable when cycloheximide was introduced in the absence of KLHL3; however, when co-transfected together with KLHL3, WNK4 abundance was strikingly reduced (Figure 3B). When we compared the effects of KLHL3 on WNK4 abundance with those of Cul3 $\Delta$ 403-459 on KLHL3 abundance, the results are remarkably similar (Figure 3C). In fact, when other groups have examined the effects of KLHL3 on WNK4 abundance, they also noted remarkably reduced WNK4 abundance in the presence of KLHL3 (31). Thus, although we cannot prove that the Cul3 $\Delta$ 403-459 and WT Cul3 have differential effects on KLHL3 synthesis, the current results, when coupled with those shown below suggest that KLHL3 is degraded more rapidly by the Cul3 mutant.

#### *Ligase-dependent and -independent effects of Cul3 $\Delta$ 403-459 on KLHL3 and WNK4.*

The Cul3 $\Delta$ 403-459 mutation has altered ubiquitin ligase activity as shown by increased ubiquitylation of KLHL3 and decreased ubiquitylation of WNK4 (14, 24). Yet, Cul3 $\Delta$ 403-459 also has ligase-independent effects, such as enhanced binding to BTB-Kelch adaptors and decreased binding to CSN subunits and cullin-associated and NEDD8-dissociated protein 1 (CAND1) (10, 14, 24). To try to better understand the anomalous effects of the Cul3 $\Delta$ 403-459 protein we generated a neddylation-deficient Cul3 $\Delta$ 403-459 double mutant, Cul3 $\Delta$ 403-459 K712R. Neddylation is generally considered to be necessary to activate CRLs. The RING subunit utilizes specific E1 and E2 enzymes to covalently attach a NEDD8 protein to lysine 712 of Cul3 (33). Cul3 $\Delta$ 403-459 K712R includes a point mutation at the neddylation-site preventing NEDD8 attachment, and rendering the construct ligase-deficient. Immunoprecipitation of FLAG-tagged Cul3 $\Delta$ 403-459 K712R showed an almost complete loss of neddylation (Figure 4A), indicating that the vast majority of Cul3 $\Delta$ 403-459

neddylation occurs at the lysine 712 residue. Co-immunoprecipitation of the different Cul3 constructs and KLHL3 showed that similar to Cul3 $\Delta$ 403-459, Cul3 $\Delta$ 403-459 K712R bound to more KLHL3 protein compared to WT-Cul3 (Figure 4B), confirming that neddylation, and therefore ubiquitin ligase activity, is not required for increased protein binding.

Ubiquitylation of the BTB-adaptor KLHL3 (Figure 4C) was greater in cells transfected with Cul3 $\Delta$ 403-459, compared to WT-Cul3, as reported previously (14). As expected, this greater KLHL3 ubiquitylation was not apparent when the neddylation deficient Cul3 $\Delta$ 403-459 K712R was transfected. Yet the current results also suggest that ubiquitin ligase activity is not fully responsible for the anomalous Cul3 $\Delta$ 403-459 activity. In cells transfected with both KLHL3 and WNK4, the abundance of KLHL3 was lower, when Cul3 $\Delta$ 403-459 was present than with WT-Cul3, consistent with increased substrate adaptor ubiquitylation and degradation by the mutant protein (Figure 4D). Cul3 $\Delta$ 403-459 also led to more WNK4 abundance than did WT-Cul3 (Figure 4D), as we, and others, have reported previously. When the ligase-deficient Cul3 $\Delta$ 403-459 K712R construct was transfected, however, the effects on KLHL3 and WNK4 were reduced compared to Cul3 $\Delta$ 403-459 toward normal (WT-Cul3) levels, (Figure 4D). Unexpectedly, the effects of Cul3 $\Delta$ 403-459 on KLHL3 were not completely removed by the ligase-deficient Cul3 $\Delta$ 403-459 K712R double mutant, indicating that the effects of Cul3 $\Delta$ 403-459 is only partially dependent on ubiquitin ligase activity.

#### *Cul3 $\Delta$ 403-459-mediated KLHL3 degradation is both proteasome- and autophagy-dependent.*

As shown above, when ubiquitin ligase activity is lacking, as in the Cul3 $\Delta$ 403-459 K712R double mutant, there is less abundance of KLHL3, however, the amount of KLHL3 abundance retained by the construct (ligase-independent degradation) is still significant. Since the neddylation-deficient construct should block the ubiquitin-proteasome degradation pathway, the remaining Cul3 $\Delta$ 403-459-mediated KLHL3 degradation could be autophagy-dependent. The proteasome is the canonical pathway for degrading ubiquitylated proteins, and it has been shown to contribute to WNK kinase degradation (14); yet there is also evidence that KLHL3 and WNK4 can be degraded via autophagy (16). To determine the pathways involved in Cul3 $\Delta$ 403-459-mediated KLHL3 degradation, we used the inhibitors MG132 and chloroquine. Proteasomal inhibition with 10  $\mu$ M MG132 had no effect on the control group, but partially suppressed Cul3 $\Delta$ 403-459-mediated KLHL3 degradation (Figure 5). Inhibition of autophagy with 100  $\mu$ M chloroquine, however, had a small but significant effect on the control group, indicating that KLHL3 is constitutively degraded via autophagy (Figure 6A). Incubation of Cul3 $\Delta$ 403-459 with chloroquine also resulted in a partial suppression of KLHL3 degradation. The percent change in KLHL3 protein abundance due to chloroquine administration was significantly different between control and Cul3 $\Delta$ 403-459 (Figure 6B), which indicates an increased autophagic degradation of KLHL3 mediated by the Cul3 mutant. To confirm these results, we treated cells with another autophagy blocker, 3-methyladenine (3-MA), an inhibitor of autophagosome formation (Figure 6C). The results closely resembled chloroquine administration further indicating autophagic KLHL3 degradation.

273 Treatment of the cells with both MG132 and chloroquine completely abolished Cul3 $\Delta$ 403-459-mediated  
274 KLHL3 degradation (Figure 6D). The data suggest that, under the conditions provided, KLHL3 is degraded by  
275 Cul3 $\Delta$ 403-459 through both the ubiquitin-proteasome pathway and the autophagy pathway. Thus, WT-Cul3 and  
276 Cul3 $\Delta$ 403-459 degrade WNK4 and KLHL3, respectively, via two different pathways. WNK4 is ubiquitylated  
277 and degraded through the proteasomal pathway. KLHL3 is degraded by both the proteasome and the autophagy  
278 pathway.

279  
280 *Increasing KLHL3 expression normalizes Cul3 $\Delta$ 403-459-mediated inhibition of WNK4 degradation in the*  
281 *presence of WT-Cul3.*

282 We suggested previously that the increased activity of Cul3 $\Delta$ 403-459 toward KLHL3 reduced the  
283 availability of KLHL3 to participate in degrading WNK kinases (14). Alternatively, others have suggested that  
284 the increased association of Cul3 $\Delta$ 403-459 with KLHL3 may sequester it, and accomplish the same effect. If  
285 the low level of KLHL3 contributes to the increase in WNK4 in Cul3 $\Delta$ 403-459 patients, then increasing the  
286 amount of KLHL3 should reduce WNK4. By increasing the amount KLHL3 DNA transfected into the cells we  
287 were able to increase KLHL3 protein abundance. In HEK293 cells that lack Cul3 (HEK293T<sup>Cul3-KO</sup>), an  
288 increase in KLHL3 protein levels caused only a slight reduction in WNK4 abundance (28%); WNK4 was still  
289 substantially higher than in cells transfected with WT-Cul3, even though KLHL3 protein abundance was similar  
290 (compare the first and last three lanes in Figure 7A). However, in HEK293 cells that contained endogenous  
291 WT-Cul3 (Figure 7B), increasing KLHL3 protein levels in cells transfected with Cul3 $\Delta$ 403-459 caused a  
292 striking reduction in WNK4 protein abundance (64%), to a value that was not significantly different from WT-  
293 Cul3 transfected cells. The data show that Cul3 $\Delta$ 403-459 itself is unable to substantially degrade WNK4, even  
294 when KLHL3 is normalized, but when Cul3 $\Delta$ 403-459 and Cul3 are both present in cells, as they are in  
295 heterozygous humans, the addition of KLHL3 normalizes degradation of WNK4. This suggests that, *in vitro*,  
296 Cul3 $\Delta$ 403-459 degrades KLHL3, preventing WT-Cul3 from binding to WNK4.

297  
298 *Cul3 $\Delta$ 403-459 exhibits a dominant effect in cells.*

299 It has been suggested that Cul3 $\Delta$ 403-459 causes FHHt by inducing functional Cul3 haploinsufficiency,  
300 (10, 24). This suggestion derives from the observation that the abundance of Cul3 $\Delta$ 403-459 is very low in a  
301 knock-in mouse model of FHHt, and also that introducing a 1:1 molar ratio of WT-Cul3 to Cul3 $\Delta$ 403-459 did  
302 not inhibit ubiquitylation of WNK4 (24). Yet Uchida and colleagues noted that mice with functional Cul3  
303 haploinsufficiency do not exhibit signs of FHHt (2), and as noted above, Cul3 $\Delta$ 403-459 may either degrade  
304 and/or sequester KLHL3, thereby exerting a dominant negative effect (10, 14). To determine whether  
305 Cul3 $\Delta$ 403-459 has dominant effects in cells, we transfected both WT-Cul3 and Cul3 $\Delta$ 403-459 together at  
306 different ratios. In the presence of constant WT-Cul3, increasing Cul3 $\Delta$ 403-459 reduced KLHL3 abundance  
307 and increased WNK4 abundance (Figure 8), and increasing WT-Cul3 in the presence of Cul3 $\Delta$ 403-459

308 increased KLHL3 and decreased WNK4. Thus, Cul3 $\Delta$ 403-459 clearly exerts a dominant effect in cultured cells;  
309 this is consistent with the autosomal dominant inheritance of FHHt type 4.

### 311 *Cul3 $\Delta$ 403-459 does not affect Keap1 and cyclin E protein abundance*

312 Because Cul3 can interact with and ubiquitinate multiple substrates through many different substrate  
313 adaptors, then it would be expected that the Cul3 $\Delta$ 403-459 mutant would affect other proteins besides KLHL3  
314 and WNK4. To better understand the effects of the Cul3 $\Delta$ 403-459 mutant, we examined three other proteins  
315 that interact with Cul3. The oxidative stress response protein, nuclear factor erythroid 2-related factor 2 (Nrf2),  
316 is another substrate of Cul3 and interacts through the BTB-Kelch substrate adaptor protein kelch-like ECH-  
317 associated protein-1 (Keap1). Unlike KLHL3, Keap1 showed no change in protein abundance when cells were  
318 transfected with Cul3 $\Delta$ 403-459 or Cul3 $\Delta$ 403-459 K712R (Figure 9). Although Keap1 was unchanged, its  
319 substrate Nrf2 showed an increase in protein abundance in Cul3 $\Delta$ 403-459 transfected cells. The amplified Nrf2  
320 was also observed in cells transfected with Cul3 $\Delta$ 403-459 K712R. Cyclin E, which is a canonical Cul3  
321 substrate and is involved in cell cycle regulation, was unchanged when transfected with Cul3 $\Delta$ 403-459 or  
322 Cul3 $\Delta$ 403-459 K712R. The results demonstrate that the Cul3 $\Delta$ 403-459 mutant has differential effects on its  
323 substrates and substrate adaptors.

## 325 **Discussion**

326 Mutations in *WNK1*, *WNK4*, *Cul3*, and *KLHL3* cause FHHt, predominantly by increasing NCC activity  
327 along the DCT, suggesting that these proteins comprise a single signaling pathway. The disease pathogenesis, in  
328 all cases, appears to result from an increase in WNK kinase abundance, either owing to enhanced transcription,  
329 or impaired degradation. While *WNK4* and *KLHL3* mutations impair the degradative arm of this pathway by  
330 disrupting the ability of WNK kinases to form complexes with KLHL3 and Cul3, the mechanisms involved in  
331 *Cul3* disease have been more difficult to unravel (17, 18, 25, 28, 29, 31, 34). We reported previously that the  
332 FHHt-mutant Cul3 ubiquitylates and facilitates KLHL3 degradation more actively than does WT-Cul3, and  
333 suggested that the mutant exhibits dominant effects (14). The dominant nature of the Cul3 $\Delta$ 403-459 mutation  
334 was very recently confirmed, using mouse models (9). Here we determined that Cul3 $\Delta$ 403-459 has an altered  
335 structure that decreases interaction with the CSN. Yet the results also discern a novel, secondary, ligase-  
336 independent autophagocytic KLHL3 degradation pathway that appears essential for the autosomal dominant  
337 phenotype.

338 The current results confirm our prior work (14), indicating that that Cul3 $\Delta$ 403-459 is hyperneddylated,  
339 when expressed in cells. Ibeawuchi and colleagues (10), in contrast, could not detect hyperneddylated  
340 Cul3 $\Delta$ 403-459 and suggested that the mutant protein is neddylation less efficiently than WT. A potential  
341 resolution to this paradox is apparent from the work of Schumacher and colleagues (24), who also documented  
342 that Cul3 $\Delta$ 403-459 is hyperneddylated in cells, but found that the neddylation process itself was less efficient.

343 They demonstrated that the defect lies in a failure of the CSN to associate with Cul3, and therefore, a failure of  
344 deneddylation. The current work confirms that Cul3 $\Delta$ 403-459 does not associate normally with the CSN (in this  
345 case, the catalytically-active JAB1 subunit), but this effect is not because the deleted amino acid sequence  
346 actually binds to JAB1. FHHt-causing Cul3 mutations lead to deletion of exon 9, which encodes the 4HB  
347 domain (Figure 1A). Min et al. (15) examined the CSN binding domain of a homologous cullin, cullin 1, and  
348 suggested that it lies within the 4HB domain. To determine whether the same domain is relevant in Cul3  
349 binding to CSN, we mapped the Cul3 domains that are required for association with JAB1. Using multiple Cul3  
350 constructs, we determined that the 4HB domain is neither necessary nor sufficient for CSN binding. Instead, the  
351  $\alpha/\beta_1$  domain, which lies adjacent to the 4HB domain, was identified as essential for association with the CSN.  
352 Since the  $\alpha/\beta_1$  domain is not directly altered by the Cul3 $\Delta$ 403-459 mutation, the results suggest that the  
353 mutation disrupts binding to the CSN through alterations in the protein folding of Cul3; this suggestion aligns  
354 with the structural modeling data of Schumacher and colleagues (24).

355 Since Cul3 $\Delta$ 403-459 does not bind efficiently to JAB1 protein we tested whether JAB1 knockdown  
356 could mimic the effects of Cul3 $\Delta$ 403-459 in cultured cells. Knockdown of JAB1 with siRNA decreased, rather  
357 than increased, WNK4 protein abundance indicating increased CRL activity (Figure 2). This suggests that JAB1  
358 knockdown alone cannot mimic Cul3 $\Delta$ 403-459 effects on WNK4; it should be noted, however, that CRL  
359 activity can differ between cell culture models and *in vivo*. The CSN positively regulates CRLs *in vivo* (22),  
360 whereas *in vitro* CRLs are negatively regulated by the CSN (36). Further, *in vivo*, experiments are needed to  
361 fully understand the effects of JAB1 inhibition.

362 The Cul3 $\Delta$ 403-459 K712R mutant prevented NEDD8 conjugation, effectively inhibiting ubiquitin ligase  
363 activity. However, Cul3 $\Delta$ 403-459 K712R, unexpectedly, still showed significant degradation of KLHL3 (Figure  
364 4D), suggesting that degradation might be due to a non-ligase effect of the mutant Cul3 $\Delta$ 403-459. The results  
365 suggest that this ligase-independent degradation of KLHL3 occurs through the autophagy pathway, as two  
366 different inhibitors of this pathway, chloroquine, and 3-MA, both prevented KLHL3 degradation. Thus, as the  
367 schematic depicts in Figure 10, Cul3 $\Delta$ 403-459 facilitates KLHL3 degradation through two different pathways.  
368 KLHL3 is ubiquitylated and degraded via the proteasome. Additionally, KLHL3 can be degraded via selective  
369 autophagy, which is stimulated by the Cul3 mutant in a ligase-independent manner. Furthermore, the results  
370 here suggest that degradation of KLHL3 contributes importantly to WNK4 accumulation in FHHt. As shown, in  
371 the absence of WT-Cul3 (in HEK293T<sup>Cul3-KO</sup> cells), Cul3 $\Delta$ 403-459 cannot degrade WNK4, even when KLHL3  
372 is abundant (Figure 7A). In cells that simultaneously express WT-Cul3 with Cul3 $\Delta$ 403-459, however, KLHL3  
373 abundance proves limiting for WNK4 degradation (Figure 7B), and thus it is the ability of the mutant Cul3 to  
374 drive KLHL3 degradation that proves essential. These observations provide substantial insight into the  
375 mechanisms of the disease, which is characterized by the presence of one WT and one mutant Cul3 allele. In  
376 this case, the protein generated from Cul3 $\Delta$ 403-459 cannot degrade WNK4, creating functional  
377 haploinsufficiency, as suggested. Yet the ability of the WT-Cul3 protein to facilitate WNK kinase degradation

378 is limited by the low abundance of KLHL3; this is maintained by enhanced proteasomal- and autophagy-driven  
379 KLHL3 degradation (Figure 10).

380 The Cul3 mutation is contained within a protein that is a part the UPS, yet Cul3 $\Delta$ 403-459 causes an  
381 increase in autophagic degradation of KLHL3 (Figure 6B). The reason for this could be due to the relationship  
382 between the two degradative pathways. The UPS and autophagy were once thought to be separate mechanisms  
383 for regulated protein turnover, however, recent work has demonstrated that the two pathways may not be  
384 independent of one another. First, inhibition of the proteasome causes an increase in autophagy (8). This is most  
385 likely due to the fact that many proteins involved in the autophagy pathway are substrates for E3 ubiquitin  
386 ligases, including CRLs, and are negatively regulated via the UPS (7). Additionally, ubiquitylated proteins can  
387 be shuttled to the autophagophore (the vesicle that ultimately binds to the lysosome) via a linker protein, such as  
388 p62 (11). These proteins connect the two pathways by binding to both ubiquitylated proteins and  
389 autophagophore-membrane proteins allowing for ubiquitylated proteins to be degraded via autophagy. p62  
390 binds to KLHL3 and mediates its degradation via autophagy when the proteasomal pathway is inhibited (16).  
391 Thus, the Cul3 mutation could lead to upregulation of p62-mediated autophagic degradation of KLHL3;  
392 moreover, the impairment of CRL substrate degradation, as shown with the Cul3 mutant, could cause activation  
393 of autophagy due to accumulation of CRL substrates that are critical for the process.

394 CRLs can associate with hundreds of substrate adaptors which can target thousands of substrates. Global  
395 deletion of the *Cul3* gene is embryonic lethal in mice (26). So, the fact that Cul3 $\Delta$ 403-459 doesn't produce  
396 widespread phenotypic effects has been perplexing. Here, we suggest that this paradox is resolved by dual  
397 effects of the Cul3 $\Delta$ 403-459 mutant protein, loss of function with respect to WNK4 degradation, and gain of  
398 function with respect to autophagocytic degradation of KLHL3. The latter effect likely contributes to the  
399 apparent tissue specificity for the disease to disrupt kidney and vascular smooth muscle. Although Cul3 $\Delta$ 403-  
400 459 avidly binds to and degrades KLHL3, its effects on other adaptor proteins are different. The substrate  
401 adaptors Bacurd1 and RhoBTB1 similarly showed higher levels of interaction with Cul3 $\Delta$ 403-459 compared to  
402 WT-Cul3 (10). Yet, Cul3 $\Delta$ 403-459 degraded RhoBTB1 less efficiently compared to WT-Cul3, and Bacurd1  
403 showed no change in abundance. Here, Keap1, also known as Kelch-like 19, like Bacurd1, did not show a  
404 change in protein abundance due to Cul3 $\Delta$ 403-459 (Figure 9). Although Cul3 $\Delta$ 403-459 has differential effects  
405 on these substrate adaptors, the effects of Cul3 $\Delta$ 403-459 on their respective substrates were similar. Analogous  
406 to WNK4, the Bacurd1 substrate RhoA, which is expressed in the vascular smooth muscle and important for  
407 arterial pressure regulation, was upregulated by Cul3 $\Delta$ 403-459 (1). Additionally, as shown above, the Keap1  
408 substrate and oxidative stress response protein, Nrf2, showed increased protein abundance (Figure 9). On the  
409 other hand, cyclin E, a protein involved in cell cycle regulation and a substrate of Cul3, was unaffected by  
410 Cul3 $\Delta$ 403-459. The data demonstrate that the altered structure of Cul3 $\Delta$ 403-459 may be sequestering adaptors  
411 in a manner that prevents normal ubiquitin ligase activity toward the substrate; yet KLHL3 may be one of only

412 a few adaptors that undergoes active degradation, providing specificity for tissues and cell types in which this  
413 protein is highly expressed.

414 All FHHt patients reported to date who harbor mutations in *Cul3* are heterozygous (4). Some have  
415 suggested that the *Cul3* $\Delta$ 403-459 protein is unstable, leading to functional haploinsufficiency of the wild type  
416 protein; according to this model, individuals with one functional *Cul3* allele should exhibit the phenotype (1,  
417 24). Yet, Uchida and colleagues generated a mouse model that lacked one *Cul3* allele, expressing approximately  
418 half as much *Cul3* protein as control mice; the mice, however, did not show any evidence of the FHHt  
419 phenotype (2). Further, Ferdaus and colleagues (9) recently showed that mice with one *Cul3* allele also lack  
420 features of FHHt, whereas mice with one mutant and one wild type allele exhibit frank hyperkalemic  
421 hypertension. While the protein derived from *Cul3* $\Delta$ 403-459 does appear to be unstable, *in vivo* (1, 24), the  
422 current results suggest that the FHHt phenotype requires a second, dominant-negative effect. In support of this,  
423 we found clear evidence for a dominant effect of *Cul3* $\Delta$ 403-459 on *Cul3* WT, when the ratio of WT to mutant  
424 construct was varied (Figure 8). This is largely consistent with the model suggested by Sigmund and colleagues,  
425 who found that *Cul3* $\Delta$ 403-459 exhibited enhanced interaction with substrate adaptors, such as KLHL3, and  
426 suggested that the mutant cullin might act in a dominant manner, despite reduced abundance, by sequestering  
427 adaptor proteins (10).

428 The results here show only a modest change in WNK4 when WT *Cul3* and *Cul3* $\Delta$ 403-459 are  
429 transfected together, and increasing the ratio of WT *Cul3* to *Cul3* $\Delta$ 403-459 further diminished the effects on  
430 WNK4 abundance. This raises questions about the direct relevance of this to the human disease, as all FHHt  
431 patients are heterozygous, with one WT and one mutant allele. Additionally, in experimental disease models,  
432 the abundance of *Cul3* $\Delta$ 403-459 is very low, relative to the wild type allele. Yet, Ferdaus and colleagues (9)  
433 showed recently that *Cul3* $\Delta$ 403-459 exerts dominant effects, *in vivo*, despite its low abundance. Thus, these  
434 limitations of using HEK293 cells imply that these hypotheses should be explored using physiologically more  
435 relevant model systems.

436 Thus, the current results clarify the consequences of deletion of exon 9 in *Cul3*, and provide novel  
437 information about how *Cul3* interacts with the CSN. They suggest that this interaction requires the  $\alpha/\beta_1$  domain,  
438 which lies next to, but is distinct from, the 4HB domain deleted in the human disease. Thus, although the  
439 deletion impairs binding of *Cul3* to the CSN, the deleted region itself does not mediate the association.  
440 Furthermore, the results stress the importance of KLHL3 in the regulation of WNK4. Our data would be  
441 consistent with an effect of the mutant *Cul3* protein to bind avidly to specific substrate adaptors, forming  
442 unstable and ineffective complexes. Additional studies will be required to further evaluate this hypothesis.  
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447 **Author Contributions**

448 R.J. Cornelius, C. Zhang, J.D. Singer, C. Yang, and D.H. Ellison designed research. R.J. Cornelius, C. Zhang,  
449 and K.J. Erspamer performed research. L.N. Agbor and C.D. Sigmund generated HEK293T<sup>Cul3-KO</sup> cells. R.J.  
450 Cornelius, C. Zhang, K.J. Erspamer, J.D. Singer, C. Yang, and D.H. Ellison analyzed data. R.J. Cornelius and  
451 D.H. Ellison wrote the paper. R.J. Cornelius, C. Zhang, K.J. Erspamer, L.N. Agbor, C.D. Sigmund, J.D. Singer,  
452 C. Yang, and D.H. Ellison edited the paper.

453

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464 **Disclosures**

465 The authors declare that they have no conflicts of interest.

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## Figure Legends

**Figure 1. CSN binds to Cul3 at the  $\alpha/\beta_1$  domain.** A) Diagram of Cul3 domain structure and schematic of the Cul3 constructs. B) Co-immunoprecipitation was performed with HEK293 cells transfected with myc-JAB1 and FLAG-tagged WT-Cul3 or Cul3 $\Delta$ 403-459 and analyzed by immunoblot. Cul3 $\Delta$ 403-459 exhibited a decreased interaction with JAB1 compared to WT-Cul3. C) The effects of Cul3 $\Delta$ 403-459 on JAB1 binding was determined by co-immunoprecipitation with N-terminal domain Cul3 constructs using anti-FLAG and analyzed by immunoblot. Co-immunoprecipitation of N-terminal domain Cul3 constructs with (1-459) and without (1-402) the 4HB domain showed no binding to JAB1. D) Segments of the Cul3 protein were generated with a GST tag and co-transfected with myc-tagged JAB1. Co-immunoprecipitation was performed using glutathione sepharose beads. Immunoblotting for JAB1 showed binding to 4HB: $\alpha/\beta_1$  and  $\alpha/\beta_1$  Cul3 constructs, but not to 4HB, WH-A: $\alpha/\beta$ :WH-B, or R1:R2:R3 Cul3 constructs. E) Co-immunoprecipitation was performed with myc-JAB1 and FLAG-tagged WT-Cul3 or Cul3 $\Delta$ 461-586 constructs. Cul3 $\Delta$ 461-586 demonstrated less binding to JAB1 protein compared to WT-Cul3. Immunoblotting for NEDD8 showed enhanced neddylation of the Cul3 $\Delta$ 461-586 construct compared to WT-Cul3. The *asterisk* indicates a nonspecific band.

**Figure 2. Effects of JAB1 inhibition on Cul3 neddylation and substrate protein abundance.** Myc-tagged KLHL3 or WNK4 was co-transfected into HEK293 cells with either JAB1 siRNA or control siRNA. The proteins were examined by immunoblot in cells with endogenous WT-Cul3. JAB1 siRNA decreased JAB1, KLHL3, and WNK4 abundance, increased NEDD8 abundance, and the neddylated form of Cul3 (top band).  $\beta$ -actin was used as a loading control.

**Figure 3. Cul3 $\Delta$ 403-459 decreases the stability of KLHL3.** A. Cycloheximide chase assay was performed with HEK293 cells co-transfected with myc-tagged KLHL3 and either FLAG-WT Cul3 or FLAG-Cul3 $\Delta$ 403-459. Due to a robust decrease in KLHL3 by the Cul3 $\Delta$ 403-459 which prevented quantification, the amount of Cul3 $\Delta$ 403-459 transfected was reduced to half of WT Cul3. Cycloheximide (100  $\mu$ g/ml) was added 36 h post transfection and cells were lysed at 0, 1, 2, 4, 8, and 24 h time points. KLHL3 protein abundance was more rapidly degraded in cells co-expressing Cul3 $\Delta$ 403-459. Right, quantitative analysis of KLHL3 protein abundance. Stain-free imaging was used as a loading control. Linear regression was used to determine the slope of each group. The differences between the slopes were significantly different ( $P < 0.001$ ). Data represent mean values  $\pm$  SEM relative to the 0 h time point. Statistical differences were examined using two-tailed unpaired Student's t test. \*  $P = 0.01$  vs WT. B. Cycloheximide chase assay was performed with HEK293 cells co-transfected with myc-tagged WNK4 in the presence or absence of KLHL3. Cycloheximide (100  $\mu$ g/ml) was added 36 h post transfection and cells were lysed at 0, 2, 4, and 6 h time points. Stain-free imaging was used as a loading control. C. Left, quantitative analysis of KLHL3; all data points are relative to WT Cul3 0 h time point.

518 Right, quantitative analysis of WNK4 protein abundance; all data points are relative to WNK4 without  
519 KLHL3 0 h time point. The effects of Cul3 $\Delta$ 403-459 on KLHL3 abundance is similar to the effects of  
520 KLHL3 on WNK4 abundance.

521 **Figure 4. Ligase-deficient Cul3 $\Delta$ 403-459 K712R double mutant blunts the effects of Cul3 $\Delta$ 403-459 on**

522 **KLHL3 and WNK4.** A) FLAG-tagged Cul3 constructs were co-transfected into HEK293 cells and  
523 immunoprecipitated using FLAG antibody. Immunoblotting for NEDD8 showed no neddylation of the  
524 K712R mutant for both WT-Cul3 and Cul3 $\Delta$ 403-459. B) Co-immunoprecipitation was performed with  
525 HEK293 cells transfected with myc-KLHL3 and FLAG-tagged WT-Cul3, Cul3 $\Delta$ 403-459, Cul3 $\Delta$ 403-  
526 459 K712R, or empty vector. Pull-down with FLAG antibodies showed that KLHL3 had more binding  
527 to Cul3 $\Delta$ 403-459 and Cul3 $\Delta$ 403-459 K712R proteins. C) A ubiquitin assay was performed for KLHL3  
528 by co-transfecting FLAG-tagged Cul3 constructs with myc-KLHL3 and HA-tagged ubiquitin.  
529 Immunoprecipitation was performed using anti-myc antibody and poly-ubiquitylation of KLHL3 was  
530 visualized by immunoblotting for anti-HA. Cul3 $\Delta$ 403-459 K712R double mutant attenuated the higher  
531 abundance of KLHL3 ubiquitylation shown with Cul3 $\Delta$ 403-459. D) Top, abundance of myc-tagged  
532 KLHL3 and WNK4 protein was examined by immunoblot in HEK293T<sup>Cul3-KO</sup> cells co-transfected with  
533 different FLAG-tagged Cul3 constructs. KLHL3 and WNK4 expression was higher and lower,  
534 respectively, in Cul3 $\Delta$ 403-459 K712R compared to Cul3 $\Delta$ 403-459. Bottom, quantitative analysis of  
535 KLHL3 and WNK4 protein abundance. Stain-free imaging was used as a loading control. Data represent  
536 individual values as well as mean  $\pm$  SEM relative to control. Statistical differences were examined by  
537 one-way ANOVA with Tukey's post hoc analysis.

538 **Figure 5. Effects of proteasome inhibition on Cul3 $\Delta$ 403-459-mediated KLHL3 degradation.** The pathway  
539 for degradation of KLHL3 by the Cul3 $\Delta$ 403-459 mutant was examined by inhibiting the proteasomal  
540 pathway with the drug MG132. HEK293 cells were co-transfected with myc-KLHL3 and either no Cul3  
541 or FLAG-Cul3 $\Delta$ 403-459. The cells were incubated with vehicle or 10  $\mu$ M MG132 for 18 hours before  
542 harvesting. Immunoblot analysis showed that inhibition of the proteasomal pathway partially blocked  
543 Cul3 $\Delta$ 403-459-mediated KLHL3 degradation. Right, quantitative analysis of KLHL3 protein  
544 abundance. GAPDH was used as a loading control. Data represent individual values as well as mean  $\pm$   
545 SEM relative to control. Statistical differences were examined by one-way ANOVA with Tukey's post  
546 hoc analysis.

547 **Figure 6. Effects of autophagy inhibition on Cul3 $\Delta$ 403-459-mediated KLHL3 degradation.** The pathway  
548 for degradation of KLHL3 by the Cul3 $\Delta$ 403-459 mutant was examined by inhibiting the autophagy  
549 pathway with the drugs chloroquine or 3-methyladenine (3-MA). HEK293 cells were co-transfected  
550 with myc-KLHL3 and either no Cul3 or FLAG-Cul3 $\Delta$ 403-459. The cells were incubated with vehicle or  
551 100  $\mu$ M chloroquine (A), or 5 mM 3-MA (C) for 18 hours before harvesting. Immunoblot analysis

552 showed that inhibition of autophagy with chloroquine or 3-MA partially blocked Cul3 $\Delta$ 403-459-  
553 mediated KLHL3 degradation, while administration of the drugs together completely eliminated KLHL3  
554 degradation. Right, quantitative analysis of KLHL3 protein abundance. B) Bar graph depicting the  
555 percent change in KLHL3 protein abundance caused by autophagy inhibition from chloroquine  
556 administration between control and Cul3 $\Delta$ 403-459 groups. D) Cells were incubated with both the  
557 proteasomal inhibitor MG132 and autophagy inhibitor chloroquine simultaneously. Administration of  
558 the drugs together completely eliminated KLHL3 degradation. GAPDH was used as a loading control.  
559 Data represent individual values as well as mean  $\pm$  SEM relative to control. Statistical differences were  
560 examined by one-way ANOVA with Tukey's post hoc analysis.

561 **Figure 7. Increased expression of KLHL3 can overcome effects of Cul3 $\Delta$ 403-459 on WNK4 in the**  
562 **presence of WT-Cul3.** HEK293T<sup>Cul3-KO</sup> cells (A) or HEK293 cells (B) were transfected with myc-  
563 WNK4 and either FLAG-tagged WT-Cul3 or Cul3 $\Delta$ 403-459 along with increasing amounts of myc-  
564 tagged KLHL3 and analyzed by immunoblot. The increased KLHL3 expression only slightly decreased  
565 WNK4 protein abundance in HEK293T<sup>Cul3-KO</sup> cells, however, HEK293 cells had a larger decrease in  
566 WNK4 which was not significantly different from WT-Cul3. Bar graphs depict quantification of KLHL3  
567 and WNK4 protein abundance. Stain-free imaging was used as a loading control. Data represent relative  
568 individual values as well as mean  $\pm$  SEM. Statistical differences were examined by one-way ANOVA  
569 with Tukey's post hoc analysis.

570 **Figure 8. WT-Cul3 and Cul3 $\Delta$ 403-459 compete for KLHL3.** Myc-tagged KLHL3 and WNK4 were co-  
571 transfected with different amounts of FLAG-tagged WT-Cul3 and Cul3 $\Delta$ 403-459. The ratio of FLAG-  
572 WT-Cul3 to Cul3 $\Delta$ 403-459 was adjusted as labeled and analyzed by immunoblot.  $\beta$ -actin was used as a  
573 loading control. Increasing the ratio of Cul3 $\Delta$ 403-459 to WT-Cul3 decreased KLHL3 and increased  
574 WNK4 protein expression. The opposite was observed when increasing the ratio of WT-Cul3 to  
575 Cul3 $\Delta$ 403-459. Bar graphs are a summary of the densitometry analysis of the blot.

576 **Figure 9. Effects of Cul3 $\Delta$ 403-459 on Keap1, Nrf2, and cyclin E.** Top, abundance of endogenous Keap1,  
577 Nrf2, and cyclin E protein was examined in HEK293T<sup>Cul3-KO</sup> cells co-transfected with different FLAG-  
578 tagged Cul3 constructs and analyzed by immunoblot. Keap1 and cyclin E showed no difference in  
579 protein abundance between the groups. Nrf2 protein levels were higher in Cul3 $\Delta$ 403-459 and Cul3 $\Delta$ 403-  
580 459 K712R transfected cells. Stain-free imaging was used as a loading control. Bottom, quantitative  
581 analysis of Keap1, Nrf2, and cyclin E protein abundance. Data represent relative individual values as  
582 well as mean  $\pm$  SEM. Statistical differences were examined by one-way ANOVA with Tukey's post hoc  
583 analysis.



584 **Figure 10. Simplified model of Cul3 $\Delta$ 403-459 effects on KLHL3 and WNK4.** KLHL3 is degraded by two  
585 separate pathways. Under normal conditions, the WT-Cul3-KLHL3 ubiquitin ligase complex (left)  
586 ubiquitylates WNK4 targeting it for degradation via the proteasome. Separate from cullin-RING-ligase  
587 activity, KLHL3 is also degraded through selective autophagy. The Cul3 $\Delta$ 403-459 FHHt mutant (right)  
588 targets KLHL3 instead of WNK4 for ubiquitylation; causing proteasomal degradation of KLHL3 while  
589 preventing WNK4 turnover. Additionally, expression of the Cul3 mutant causes enhanced autophagic-  
590 mediated degradation of KLHL3. The lower levels of KLHL3 through both proteasomal and autophagic  
591 degradation prevent WT-Cul3 from interacting with WNK4, leading to an increase in WNK4 protein  
592 abundance.

597 **Table 1.** Antibodies used for Western blot

Figure	Target	Antibody Name	Source	1° Dilution	2° Dilution
1E,2	JAB1	JAB1 FL-334	Santa Cruz, SC-9074	1:1,000, 1h at RT	1:5,000
1C,1D	JAB1	JAB1 6C3.38	Thermo Scientific	1:2,000, 1h at RT	1:5,000
1D	GST	GST B-14	Santa Cruz, SC-138	1:1,000, 1h at RT	1:2,500
1,2,3,4,5,6,7,8	Myc	c-Myc	Sigma Aldrich, M5546	1:5,000, 1h at RT	1:10,000
1E,2,4A	NEDD8	NEDD8 19E3	Cell Signaling 2754	1:1,000, o/n at 4°C	1:2,500
2,4D	Cul3	Cul3	Cell Signaling 2759	1:1,000, o/n at 4°C	1:2,500
2,8	β-actin	β-actin	Abcam Ab8227	1:5,000, 1h at RT	1:2,500
1,4,5,6,7,8,9	FLAG	FLAG M2	Sigma Aldrich, F3165	1:10,000, 1h at RT	1:10,000
4C	HA	HA.11	Covance MMS-101P	1:1,000, 1h at RT	1:10,000
5,6	GAPDH	GAPDH	Santa Cruz, SC-20357	1:1,000, 1h at RT	1:2,500
9	Keap1	Keap1	Abcam Ab139729	1:1,000, o/n at 4°C	1:2,500
9	Nrf2	Nrf2 H-300	Santa Cruz, SC-13032	1:1,000, o/n at 4°C	1:2,500
9	Cyclin E	Cyclin E HE12	Santa Cruz, SC-247	1:1,000, o/n at 4°C	1:2,500

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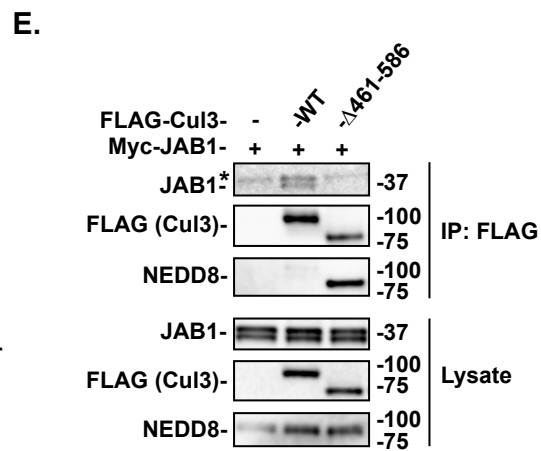
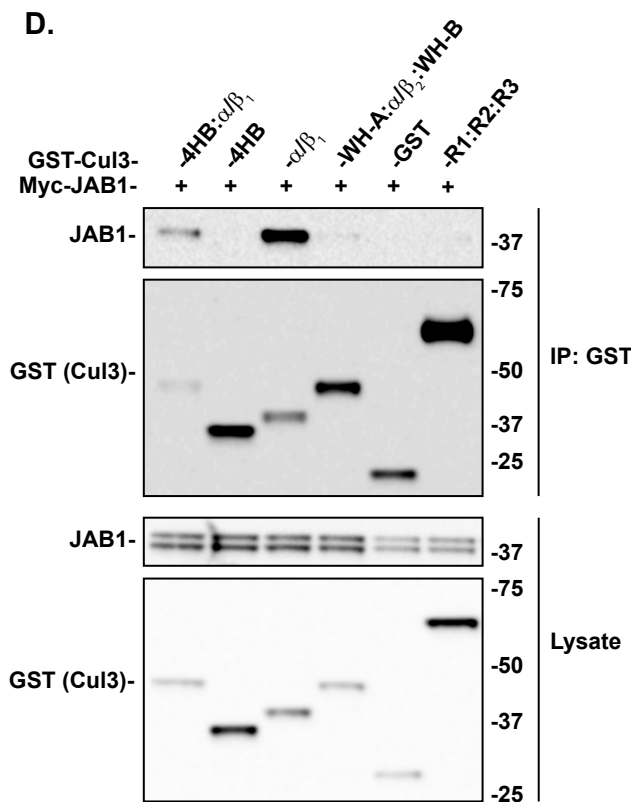
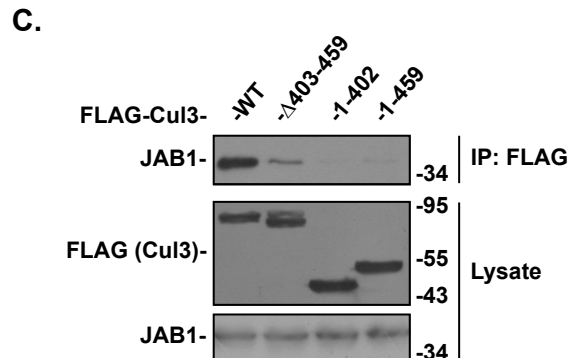
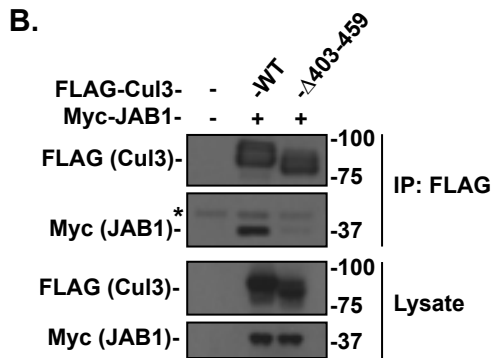
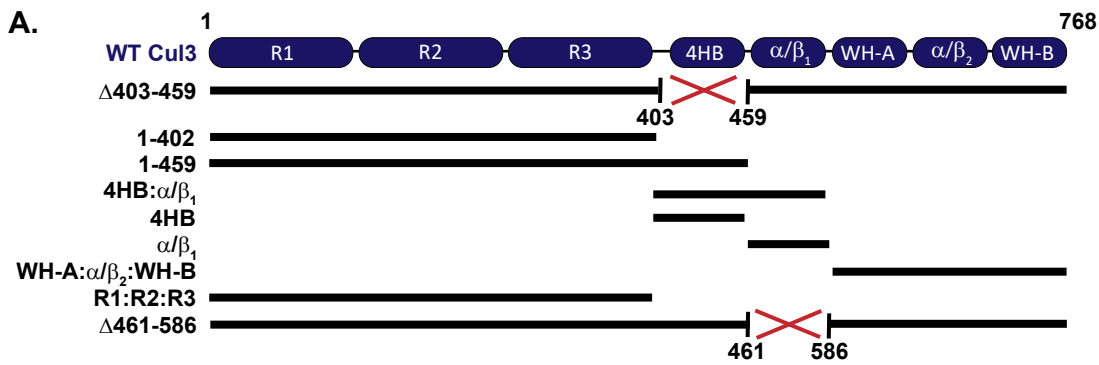
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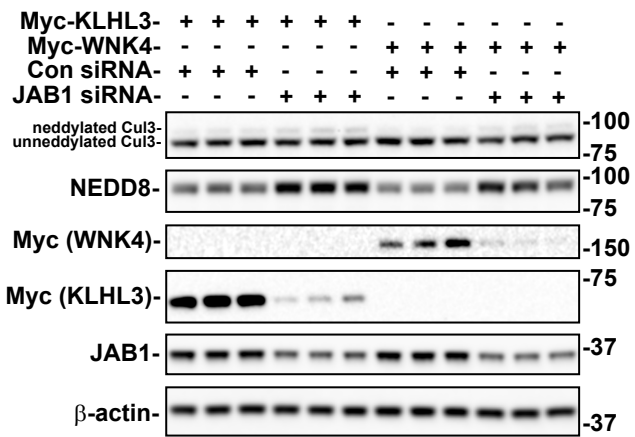
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Abbreviations: App, application; RT, room temperature; o/n, overnight; JAB1, jun activation domain-binding protein-1; GST, glutathione S-transferase; NEDD8, neuronal precursor cell expressed developmentally down-regulated protein 8; Cul3, cullin 3; Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2.

**Figure 1**

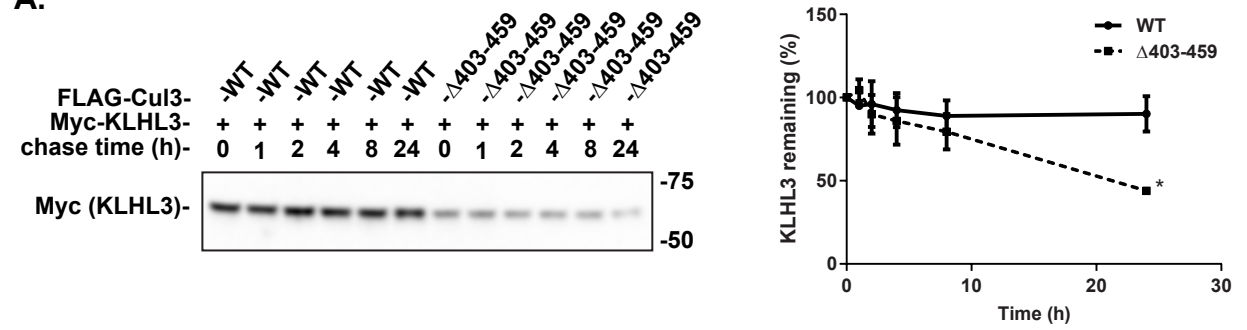


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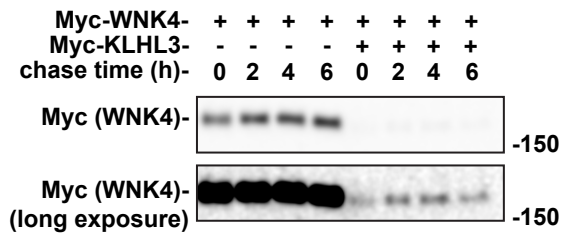


**Figure 3**

**A.**



**B.**



**C.**

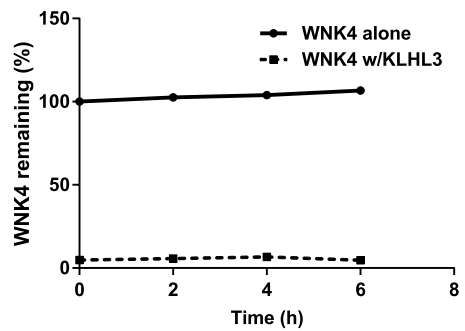
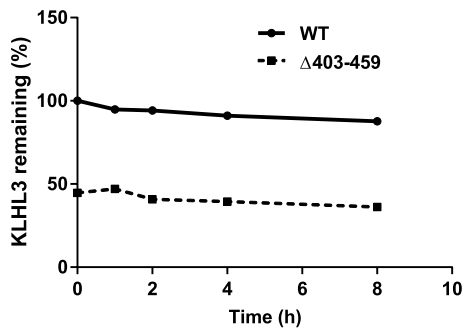
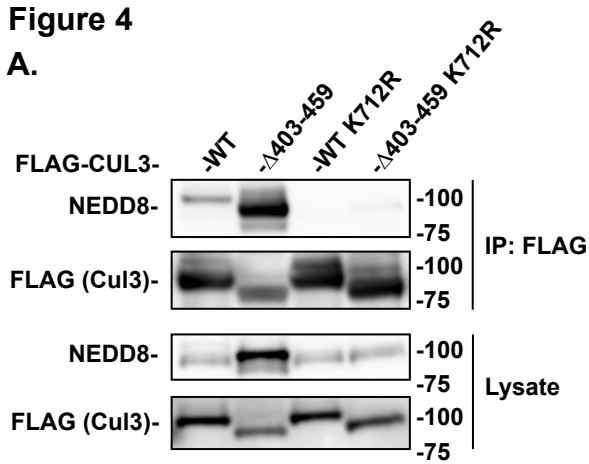
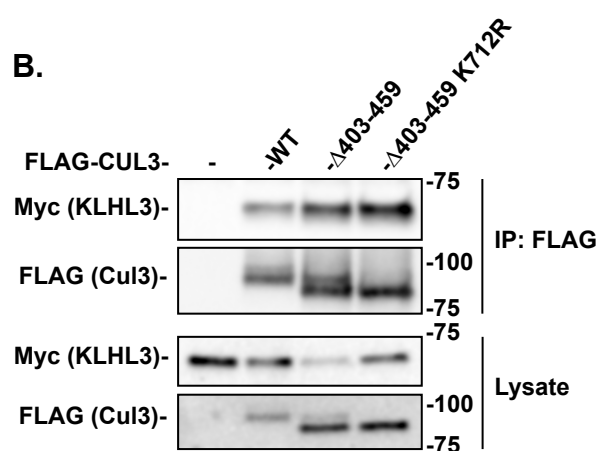


Figure 4

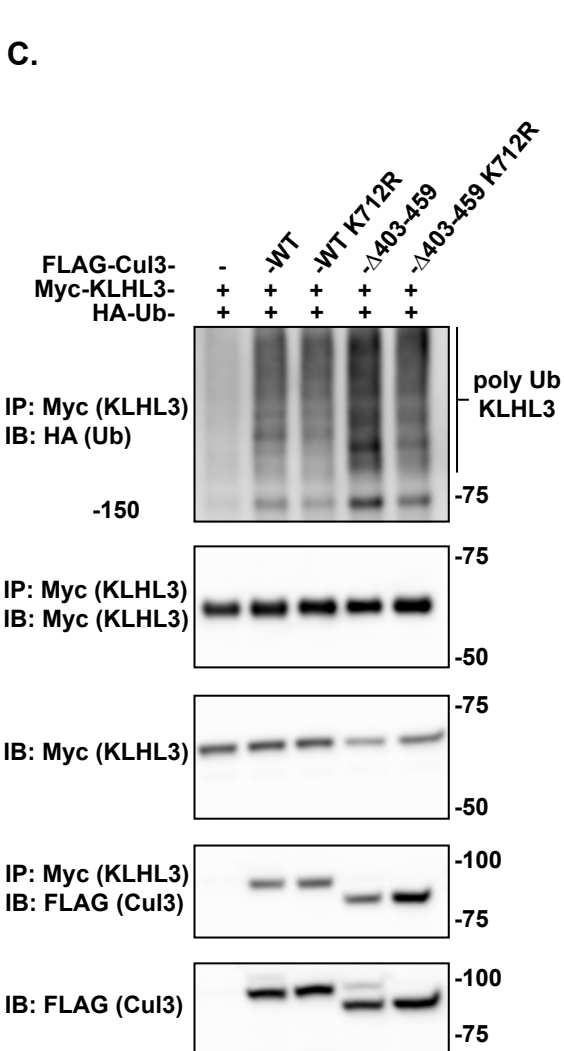
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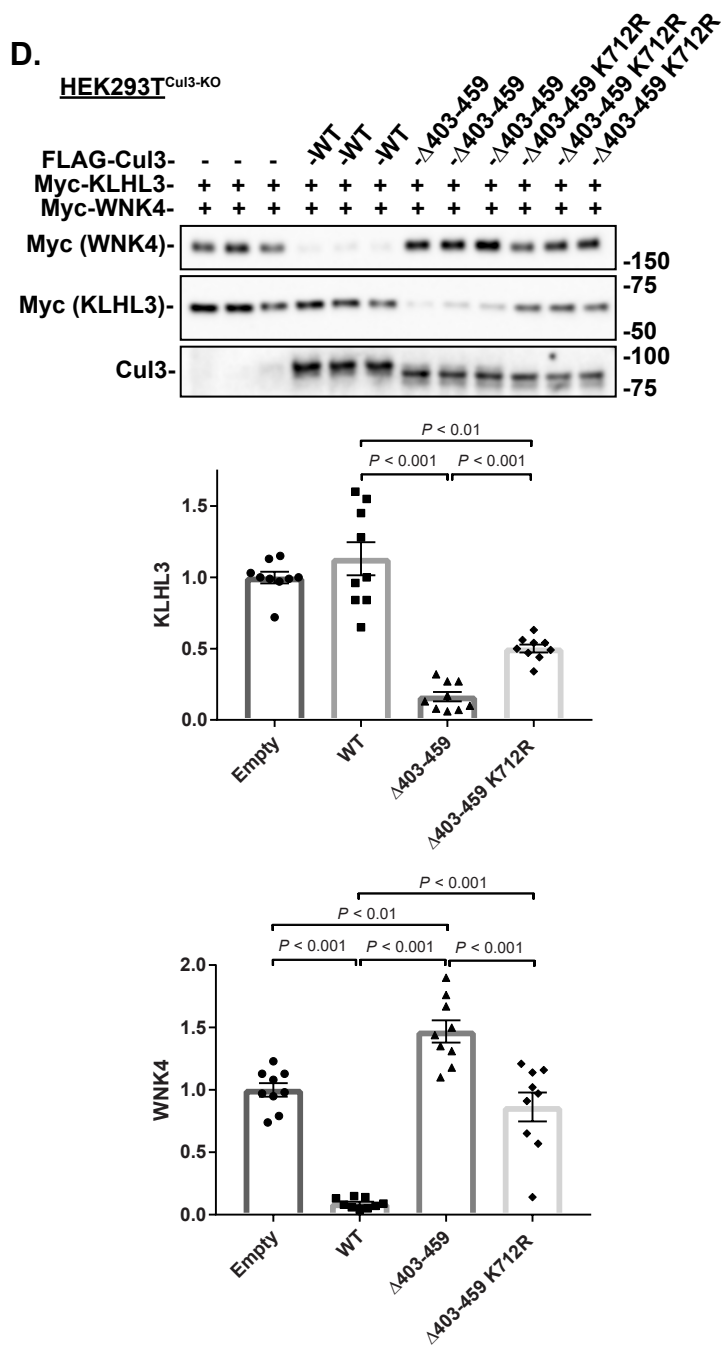
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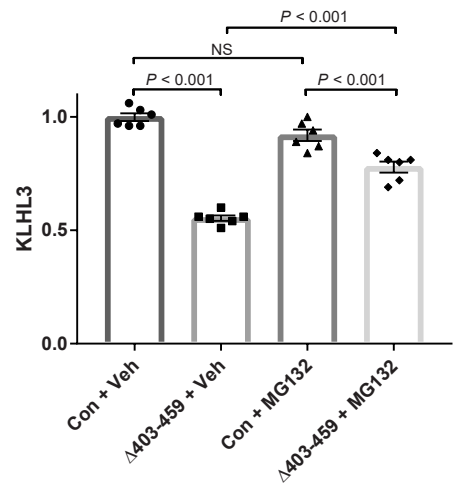
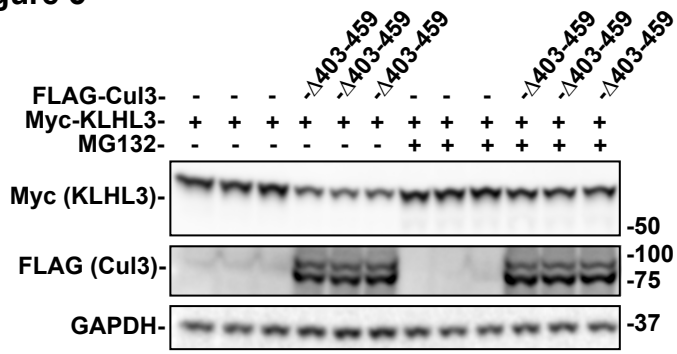
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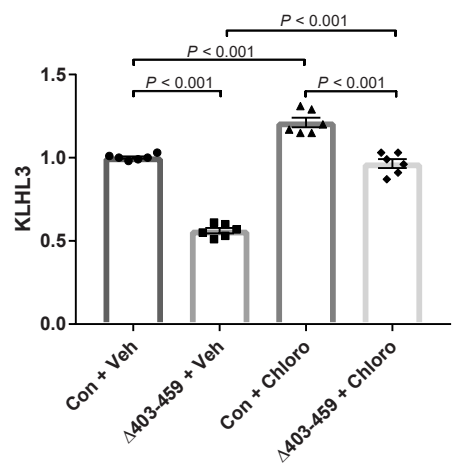
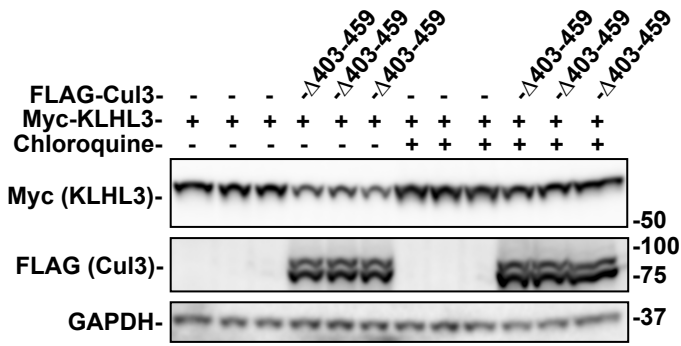


D.



**Figure 5**



**A.****B.**

## Autophagy-mediated KLHL3 degradation

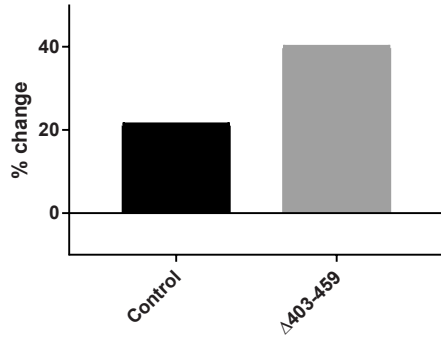
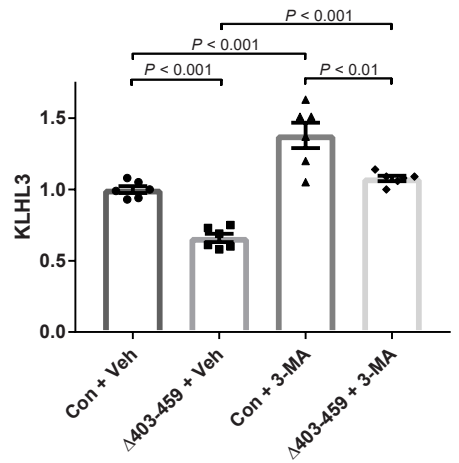
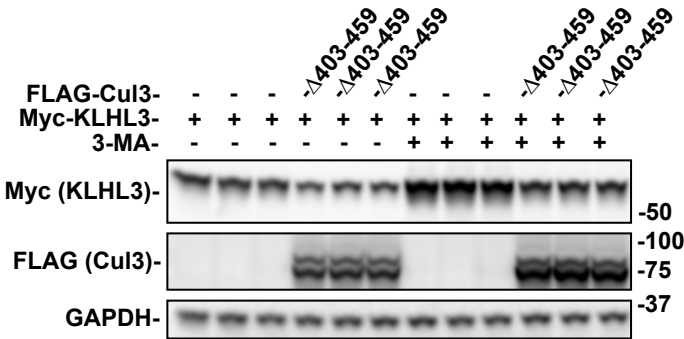
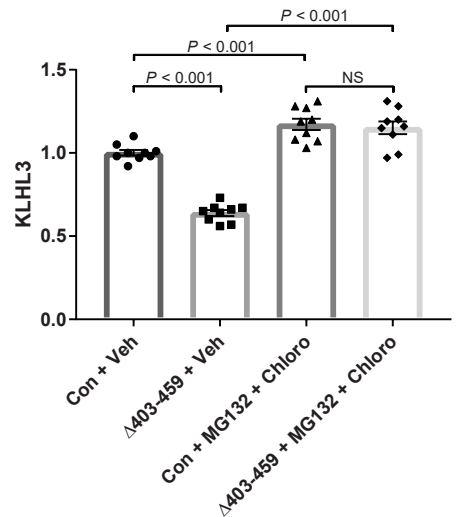
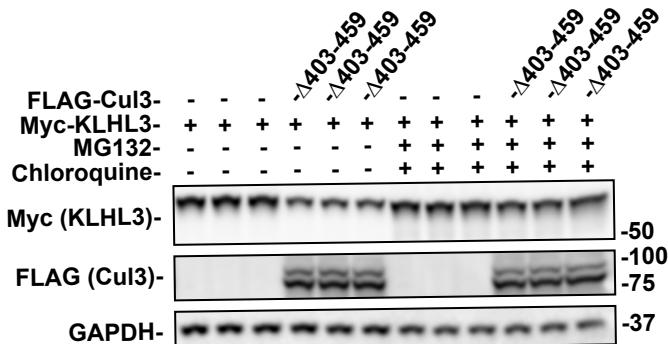
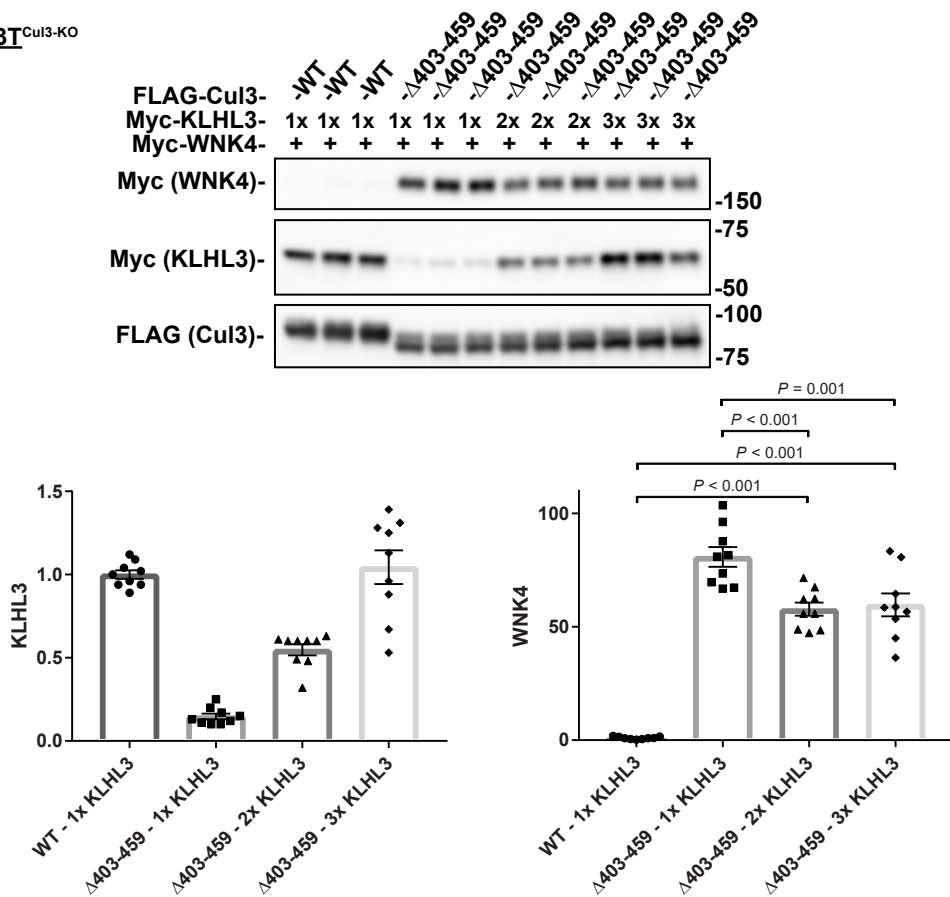
**C.****D.**

Figure 6

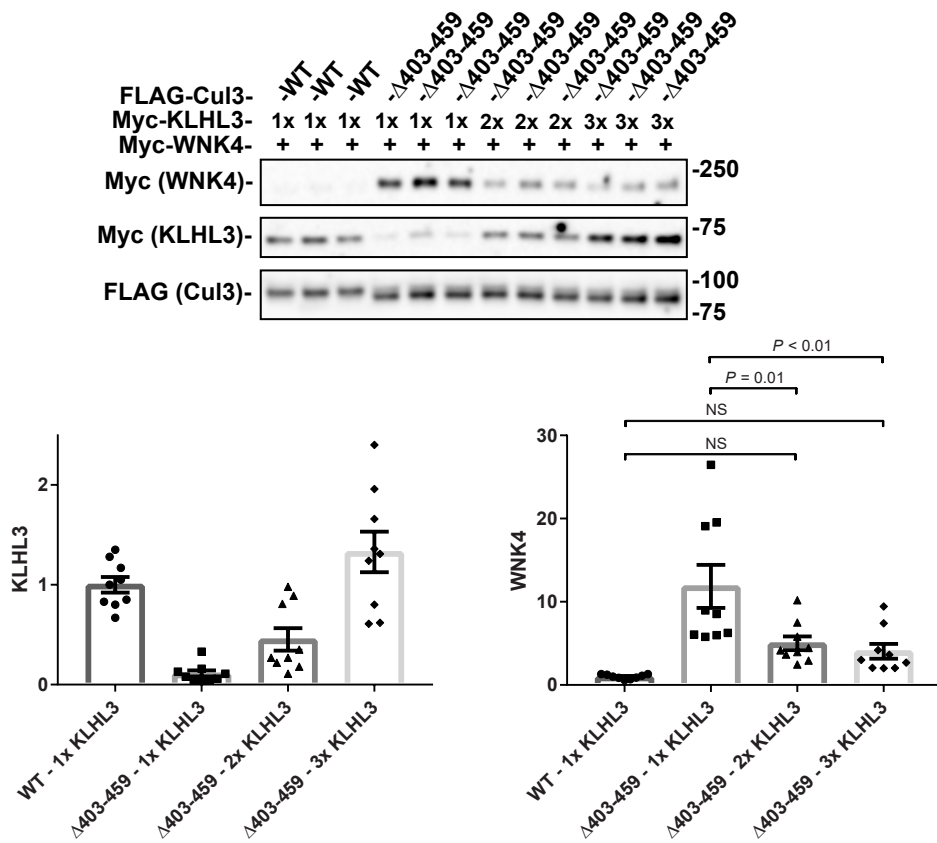


**Figure 7**

**A. HEK293T<sup>Cul3-KO</sup>**



**B. HEK293**



**Figure 8**

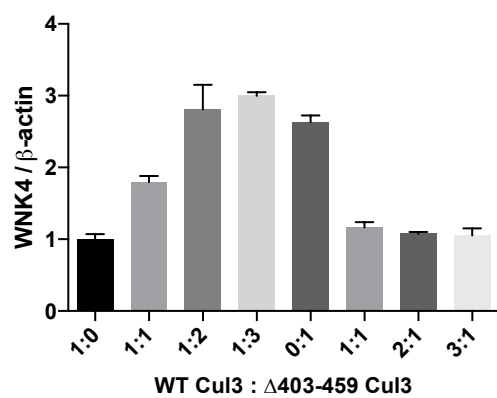
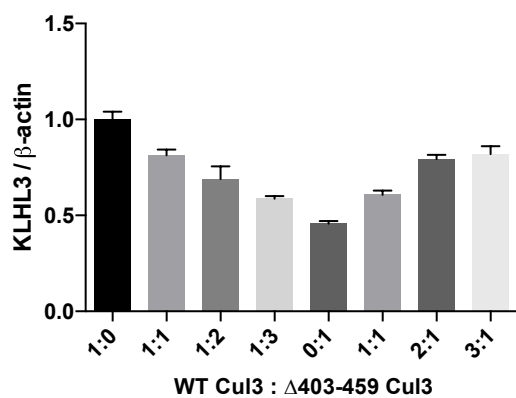
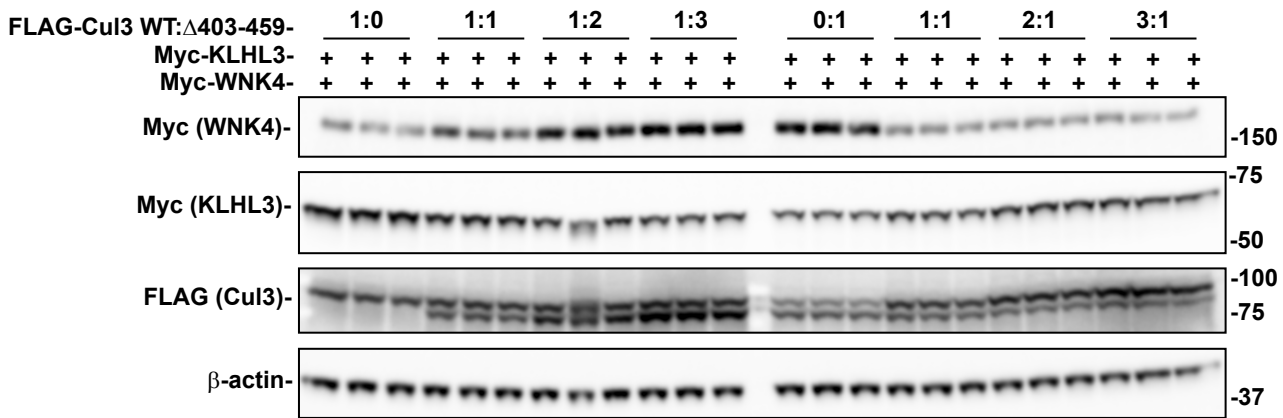
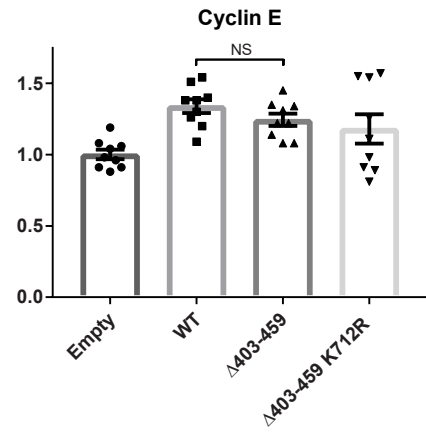
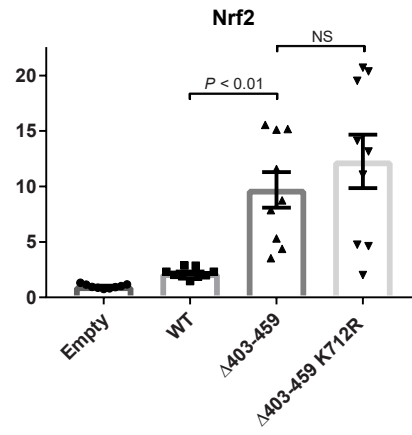
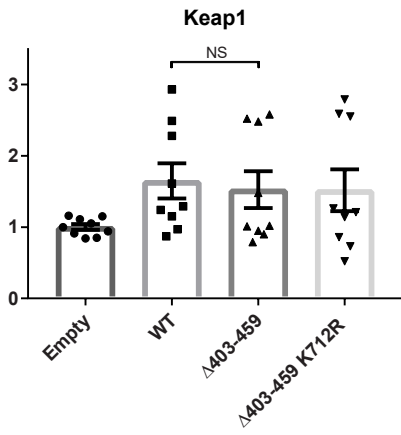
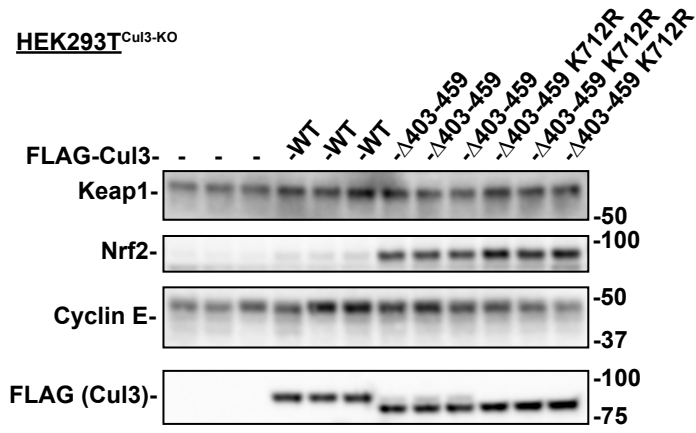


Figure 9

HEK293T<sup>Cul3-KO</sup>



**Figure 10**

