Mutations in kelch-like 3 and cullin 3 cause hypertension and electrolyte abnormalities

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Hypertension affects one billion people and is a principal reversible risk factor for cardiovascular disease. Pseudohypoparathyroidism type II (PHAII), a rare Mendelian syndrome featuring hypertension, hyperkalaemia and metabolic acidosis, has revealed previously unrecognized physiology orchestrating the balance between renal salt reabsorption and K+ and H+ excretion. Here we used exome sequencing to identify mutations in kelch-like 3 (KLHL3) or cullin 3 (CUL3) in PHAII patients from 41 unrelated families. KLHL3 mutations are either recessive or dominant, whereas CUL3 mutations are predominantly de novo. CUL3 and BTB-domain-containing kelch proteins such as KLHL3 are components of cullin–RING E3 ligase complexes that ubiquitinate substrates bound to kelch propeller domains. KLHL3 mutations are clustered in short segments within the kelch propeller and BTB domains implicated in substrate and cullin binding, respectively. Diverse CUL3 mutations all result in skipping of exon 9, producing an in-frame deletion. Because dominant KLHL3 and CUL3 mutations both phenocopy recessive loss-of-function CUL3 mutations, they may abrogate ubiquitination of KLHL3 substrates. Disease features are reversed by thiazide diuretics, which inhibit the Na+–Cl− cotransporter in the distal nephron of the kidney; KLHL3 and CUL3 are expressed in this location, suggesting a mechanistic link between KLHL3 and CUL3 mutations, increased Na+–Cl− reabsorption, and disease pathogenesis. These findings demonstrate the utility of exome sequencing in disease gene identification despite the combined complexities of locus heterogeneity, mixed models of transmission and frequent de novo mutation, and establish a fundamental role for KLHL3 and CUL3 in blood pressure, K+ and pH homeostasis.

A small number of genes causing Mendelian forms of hypertension have been identified, establishing the role of increased renal salt reabsorption in its pathogenesis16–19. The study of PHAII has identified a physiological mechanism that orchestrates activities of diverse electrolyte flux pathways, allowing maximal salt reabsorption in response to aldosterone when angiotensin II (AII) is elevated, as in settings of reduced intravascular volume (hypovolaemia), versus maximal potassium secretion in settings of hyperkalaemia, in which aldosterone is elevated without changes in AII1. The role of WNK kinases in this process was revealed by discovery of their mutation in a small fraction of PHAII kindreds11. Dominant gain-of-function mutations in WNK4 or WNK1 lead to constitutively increased salt reabsorption in the distal nephron regardless of volume status, resulting in hypertension, and inhibition of K+ secretion despite marked hyperkalaemia13–15.

We studied a cohort of 52 PHAII kindreds, including 126 affected subjects with renal hyperkalaemia and otherwise normal renal function; hypertension and acidosis were present in 71% and 82%, respectively. There was wide variation in disease severity and age of clinical presentation (Supplementary Figs 1 and 2). Mutations in WNK1 or WNK4 were present in only seven of these kindreds (13%). Those without WNK mutations had only 2.0 ± 1.4 affected members, complicating mapping efforts.

Exome sequencing of eleven unrelated PHAII index cases without WNK mutations was performed. Index cases and affected relatives (five trios and one quartet) were also subjected to genome-wide SNP genotyping. Tabulation of high quality novel protein-altering variants revealed 124 genes with three or more variants, 50 with four or more, and 23 with five. Concurrent analysis of linkage among the multiplex families was used to prioritize loci harbouring variants that co-segregated with

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disease; this identified 28 genes with novel protein-altering variants that co-segregated with disease in two or more multiplex families. This revealed KLHL3 as a strong candidate, with novel KLHL3 mutations comprising five alleles in three kindreds, all of which co-segregated with the trait. These include one kindred in which affected members are homozygous for a nonsense mutation (W470X), one in which affected members are compound heterozygotes for two missense mutations (F322C and S410L), and one segregating a heterozygous missense mutation (R528H). As a confirmation of significance, Fisher’s exact test was used to compare the prevalence of novel protein-missense mutation (R528H). As a confirmation of significance, mutations (F322C and S410L), and one segregating a heterozygous affected members are compound heterozygotes for two missense mutations (in all genes in PHAII cases versus 699 control exomes). KLHL3 was sequenced in all PHAII index cases, identifying novel mutations in 24 (Fig. 1a, b and Supplementary Figs 3 and 4). Nearly all are at positions conserved among orthologues (Supplementary Fig. 5). Sixteen kindreds have heterozygous mutations that co-segregate with the trait under a dominant model (log of odds (lod) score 6.9, <−2 under other models). In contrast, eight index cases inherited mutations in both KLHL3 alleles. In these kindreds, affected members are confined to siblings of index cases who inherited the same two mutations, whereas unaffected relatives inherited zero or one mutation (lod score 4.3 for a recessive model, <−2 for other models). Recessive transmission has not been previously described for PHAII. Consistent with two modes of transmission, subjects with dominant KLHL3 mutations had significantly higher serum K⁺ levels (6.2 ± 0.6 mM)

**Figure 1 | Recessive and dominant KLHL3 mutations in PHAII kindreds.** a, b, Representative kindreds demonstrating recessive (a) and dominant (b) KLHL3 mutations (all 24 kindreds are shown in Supplementary Figs 3 and 4). Affected, unaffected and phenotype-undetermined subjects are denoted by black, white and grey symbols, respectively. Recessive (light blue) and dominant (pink) mutations are shown; recurrences are indicated by numbers. c, CRL schematic, comprising a BTB-containing kelch protein (KLHL3), CUL3 and a ubiquitin (Ub)-transfer-mediating RING protein, with substrate bound via the kelch propeller. The complex is shown as a dimer.
than heterozygotes for recessive mutations (4.8 ± 0.6 mM) (P < 10^{-4}, Student’s t-test; normal range 3.5–5.0 mM). These findings establish that PHAII can be caused by either recessive or dominant KLHL3 mutations. Importantly, we infer that mutations in dominant kindreds are probably dominant-negative, because they phenocopy the features of recessive disease.

KLHL3 contains an amino-terminal BTB domain, a BACK domain, and carboxy-terminal kelch-like repeats that form a six-bladed β-propeller structure\(^6\)\(^{-8}\) (Fig. 1c–e). There are over 50 BTB-containing kelch (BTB-kelch) genes in humans\(^4\); their propeller domains bind substrate proteins, promoting substrate ubiquitination via interaction of the BTB domain with CUL3, a component of a cullin–RING E3 ubiquitin ligase (CRL)\(^3\)\(^{-5}\)\(^,\)\(^6\). Ubiquitination serves diverse functions, including targeting proteins for proteasomal degradation as well as non-degradative roles such as modulation of protein activity, interaction and localization\(^7\)\(^,\)\(^8\).

Whereas recessive KLHL3 mutations are distributed throughout the encoded protein, dominant KLHL3 mutations show marked clustering (Fig. 1c). Nine of sixteen dominant mutations alter one of the last four amino acids of the six ‘d–a’ loops that connect the outermost (‘d’) β-strand of one kelch propeller blade to the innermost (‘a’) β-strand of the next blade. Two others are in ‘b–c’ loops. These dominant PHAII mutations lie near the hub of the propeller (Fig. 1d) at or near sites implicated in substrate binding in paralogues\(^9\) (Supplementary Fig. 5).

Three other dominant mutations cluster within the BTB domain, at or near sites implicated in cullin binding in paralogues\(^5\). We infer that dominant mutations in KLHL3 probably impair binding either to specific substrates or to CUL3.

After accounting for KLHL3, WNK1 and WNK4 mutations, 21 PHAII kindreds without mutations remained. We considered the presumed functional partner of KLHL3, CUL3 (Fig. 1e), as a potential candidate. Among PHAII exomes, novel heterozygous CUL3 variants were suggested in two. Sequencing CUL3 in all index cases identified 17 with novel heterozygous mutations, all in cases without KLHL3, WNK1 or WNK4 mutations (Fig. 2a and Supplementary Fig. 6). Eight of these mutations were documented to be de novo, providing overwhelming evidence that these mutations are disease-causing. CUL3 mutations all cluster in sites implicated in splicing of exon 9, including the intron 8 splice acceptor (n = 4), the intron 9 splice donor (n = 5), the putative intron 8 splice branch site (n = 3), and a putative splice enhancer in exon 9 (n = 3, within a TTAGA(T/A)) splice enhancer consensus sequence\(^1\(^{\text{a}}\)\(^{\text{b}}\)\(^{\text{c}}\)\(^{\text{d}}\)\(^{\text{e}}\)\(^{\text{f}}\)\(^{\text{g}}\)\(^{\text{h}}\)\(^{\text{i}}\)\(^{\text{j}}\)\(^{\text{k}}\...(Fig. 2b).

To test the impact of these mutations on splicing, CUL3 genomic DNA spanning exon 8 to exon 10 containing either wild-type sequence or one of nine PHAII mutations was cloned and expressed in HEK293 cells, and the spliced RNA products were analysed. Whereas the wild-type sequence produces a properly spliced product containing all three exons, each of the mutants produces a predominant product that skips exon 9, joining exon 8 to exon 10 (Fig. 2c, d). This results in an in-frame 57 amino acid deletion (residues 403–459) in the segment linking the

Figure 2 | Dominant CUL3 mutations in PHAII kindreds cause skipping of exon 9. a, Representative kindreds demonstrating CUL3 mutations, depicted as in Fig. 1 (all 17 kindreds are shown in Supplementary Fig. 6). in8, intron 8; ex9, exon 9; int5, intron 9. Positions are numbered relative to splice sites and the first base of the exonic splice (ES) enhancer. b, CUL3 mutation locations. Consensus splicing sequences\(^1\(^{\text{a}}\)\(^{\text{b}}\)\(^{\text{c}}\)^{\text{d}}\(^{\text{e}}\)^{\text{f}}\(^{\text{g}}\)^{\text{h}}\(^{\text{i}}\)^{\text{j}}\(^{\text{k}}\)... and corresponding wild-type CUL3 sequences are shown; invariant bases (green) and consensus homology (yellow) are indicated. Mutations are shown in red; recurrences are indicated by numbers. Ins.G, insertion of a G (guanine). c, Reverse transcription–polymerase chain reaction (RT–PCR) of spliced RNA. Wild-type CUL3 constructs produce a single product including exons 8, 9 and 10 (844 bp); all nine mutants tested produce a predominant product that skips exon 9 (673 bp). d, Representative RT–PCR sequences. The wild-type construct produces complementary DNA with properly spliced junctions between exons 8–9 (top) and 9–10 (middle), whereas the mutant construct (splice donor g(+1)c) produces cDNA joining exon 8 to exon 10 (bottom).
BTB-binding and RING-binding domains of CUL3. The fact that CUL3 mutations phenocopy recessive KLHL3 mutations suggests that they abrogate CUL3 function at KLHL3 targets. As with PHAII caused by WNK1 and WNK4 mutations, virtually all patients with KLHL3 and CUL3 mutations have been treated with thiazide diuretics, inhibitors of the Na–Cl cotransporter (NCC), resulting in correction of phenotypic abnormalities. WNK4 regulates the activities of NCC13,14,16, the epithelial Na⁺ channel ENaC17, and the K⁺ channel ROMK15, and is co-expressed with these proteins in the renal distal convoluted tubule (DCT) and collecting duct15,19,20. Staining mouse kidney sections with specific antibodies demonstrated that KLHL3 is predominantly expressed in the DCT and collecting duct, with apical localization in the DCT (Fig. 3). CUL3 is ubiquitously expressed and is in all nephron segments, with particularly high expression in the proximal tubule, but also in the DCT and collecting duct (Supplementary Fig. 7). These findings are consistent with both proteins having a role in the regulation of salt and electrolyte homeostasis in the distal nephron.

There are highly significant differences in phenotypic severity among PHAII patients with mutations in different genes (Table 1 and Supplementary Table 4). Subjects with CUL3 mutations presented at much younger ages than those with mutation in KLHL3, WNK1 or WNK4, had significantly more severe hyperkalaemia and metabolic acidosis, and were far more likely to have hypertension before age 18 (others commonly develop hypertension at later ages). The majority of subjects with CUL3 mutations demonstrated failure to thrive or growth impairment. These observations, in conjunction with the high rate of CUL3 de novo mutation, support impairment of reproductive fitness. Among the other mutant loci, there remain significant differences in disease severity (rank order recessive KLHL3 > dominant KLHL3 > WNK4 > WNK1).

KLHL3 and CUL3 mutations account for 79% of kindreds in our cohort. Gene identification was complicated by the combined effects of locus heterogeneity, two modes of transmission at one locus, and few informative meioses. Many so far unsolved Mendelian traits may have similar complexities. Use of control exomes as comparators for analysis of mutation burden may be broadly applicable to discovery of such loci.

The most parsimonious mechanism of KLHL3 and CUL3 mutations is that they abrogate ubiquitination of targets normally bound by CUL3, activity that is required for normal modulation of renal salt, K⁺ and HCO₃⁻ handling in response to physiological challenge; this speculation will require biochemical verification. The fact that recessive mutations in KLHL3 cause PHAII without other diverse effects implies either that KLHL3 targets are highly restricted to the renal salt and electrolyte pathway, or that loss of KLHL3 function at other targets can be compensated by other loci. BTB-kelch–CUL3 CRLs can act as dimers, with two substrate-binding domains capable of engaging the same target molecule21. This suggests a potential mechanism to explain dominant-negative effects of KLHL3 and CUL3 mutations.

CUL3-based CRLs participate in a wide range of critical cellular processes via binding diverse BTB-domain-containing proteins15. CUL3 mutations affecting all or many of these activities would undoubtedly produce very broad phenotypes. CUL3 mutations in PHAII merely phenocopy the effects of loss of KLHL3, suggesting that they selectively abrogate function at KLHL3 targets. The stereotypic consequences of CUL3 mutations, all deleting 57 amino acids in a region linking the BTB-binding and RING-binding domains, support such a specific effect. Consistent with this possibility, introduction of a flexible linker sequence to this region of CUL1 leaves substrate protein binding and ubiquitin polymerization intact, but nonetheless abolishes ubiquitination of a normal substrate21.

Table 1 | PHAII phenotypes, stratified by genotype.

| Mutant gene | No. of kindreds | No. of affecteds | Dx/Ref age* | K⁺ (mM) (nl 3.5–5.0 mM)† | HCO₃⁻ (mM) (nl 22–26 mM)† | Hypertension at age 18 (%)* † |
|-------------|----------------|-----------------|-------------|--------------------------|---------------------------|----------------------------|
| CUL3        | 17             | 21              | 9 ± 6       | 7.5 ± 0.9                | 7.7 ± 1.6                 | 14                        |
| KLHL3 recessive | 16             | 14              | 26 ± 14     | 6.8 ± 0.5                | 17.6 ± 1.5                | 14                        |
| KLHL3 dominant | 16             | 14              | 24 ± 18     | 6.2 ± 0.6                | 17.2 ± 2.5                | 17                        |
| WNK4        | 5              | 15              | 28 ± 18     | 6.4 ± 0.7                | 20.8 ± 2.3                | 10                        |
| WNK1        | 2              | 23              | 36 ± 20     | 5.8 ± 0.8                | 22.4 ± 4.6                | 13                        |

Dx/Ref age, age at diagnosis or referral. K⁺, serum potassium; HCO₃⁻, serum bicarbonate; nl, normal range. Hypertension at age 18 (%), percentage of affected patients diagnosed with hypertension by age 18. Values for Dx/Ref age, K⁺ and HCO₃⁻ are means ± standard deviations. Significance of differences among genotype classes was calculated by ANOVA (Dx/Ref age, K⁺, HCO₃⁻) or Fisher’s exact test (hypertension). *P < 0.0002. †P < 0.0001.
Thiazide diuretics correct abnormalities in virtually all PHAII subjects; similar correction is seen in a mouse model of PHAII with either thiazides or genetic ablation of NCC. These findings suggest that increased NCC activity is likely to be a common pathogenic mechanism. Co-expression of KLHL3 and CUL3 with NCC in the DCT, and evidence that NCC is ubiquitinated is consistent with this notion. ROMK and the H+ ATPase are respectively required for net renal K+ secretion and H+ secretion and are also likely targets, although their activity is expected to be decreased, rather than increased, in PHAII. Another thiazide-sensitive Na–Cl cotransport pathway in the collecting duct has recently been described, suggesting an additional potential target. Whether a KLHL3–CUL3 CRIL acts directly or indirectly on these targets, whether they alter delivery of NCC and other targets to, or retrieval from, the plasma membrane, and what upstream pathways regulate this activity is unknown. Similarly, whether KLHL3–CUL3 and WNKs operate within the same or different pathways is presently unknown; it is of interest that segments of NRF2 that interact with the kelch protein family are highly acidic, akin to the domain of WNK4 that is mutated in PHAII.

These findings demonstrate previously unrecognized roles for KLHL3 and CUL3. Understanding the upstream regulators and downstream targets of KLHL3–CUL3 activity will provide further insight into the mechanisms underlying maintenance of blood pressure and electrolyte homeostasis in response to diverse environmental challenges.

METHODS SUMMARY
A cohort of 52 PHAII kindreds comprising 126 affected subjects was ascertained, characterized and recruited for study. Index cases of eleven kindreds were chosen for whole exome capture and sequencing, and novel variants were identified. These findings demonstrate previously unrecognized roles for KLHL3 and CUL3. Understanding the upstream regulators and downstream targets of KLHL3–CUL3 activity will provide further insight into the mechanisms underlying maintenance of blood pressure and electrolyte homeostasis in response to diverse environmental challenges.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions L.M.B., M.C., K.A.C. and R.P.L. designed experiments and analysed data. L.M.B., C.J.N.-W. and J.R.T. performed experiments. A.F., H.R.T., G.C., M.L., R.D.G., B.A.S., A.P., M.J.V., S.M.O., M.F., K.E.K., J.R.T., J.R.S., K.X.M.-K., C.C.P., S.K.A., I. W., I.D.D., S.B.D., A.B., J.J.F., C.W.B., T.E.H., R.D.N., H.T., T.R.P.C., M.P., D.B., M.S., P.V., J.W.F., M.R., F.T., H.Z.A.-S., J.R., A.G.G. and B.G. recruited PHAII subjects and families. R.B. and S.M.M. directed the information technology and DNA sequencing infrastructure. L.M.B. and R.P.L. wrote the manuscript.

Author Information mRNA and protein sequences are available at NCBI under accession numbers NM_017415.2 and NP_059111.2 (KLHL3), NM_003590.3 and NP_003811.1 (CUL3); mutation data is available at dbSNP under batch accession 1056535. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to R.P.L. (richard.lifton@yale.edu).
METHODS

Study subjects. Index cases were referred for PHAII. Patients and participating family members provided consent to a study protocol approved by the Yale Human Investigation Committee. Control exomes were 699 unrelated subjects of European ancestry without hypertension, sequenced as part of diverse gene discovery projects. Genomic DNA was isolated from venous blood using standard methods.

Exome capture, sequencing and variant calling. Genomic DNA from eleven PHAII index cases and 699 controls was captured on NimbleGen 2.1M human exome arrays (Roche) and sequenced on the Illumina GenomeAnalyzer as previously described. Reads were mapped to the reference genome (hg18) using Maq and genotypes of targeted bases were called with SAMTools. Variants found in dbSNP v.130 or 1000 Genomes databases were excluded from further analysis. Remaining variants were considered ‘novel’ and annotated for impact on the encoded protein, conservation and expression. Aligned reads were viewed with the Integrative Genomics Viewer. Among PHAII cases, 94.2% of targeted bases were read by eight or more independent reads; sensitivity and specificity of heterozygous calls were estimated at 93.7% and 99.9% by comparison to Illumina SNP genotyping. Among controls, 94.4% of targeted bases were read by eight or more independent reads; sensitivity and specificity of heterozygous calls were estimated at 94.5% and 99.8%. Sanger sequencing of 212 novel variants from controls with a SAMtools quality score of ≥100 demonstrated validation in 211 and amplification failure in 1, supporting high specificity of variant calls.

SNP genotyping and linkage analysis. For the 11 PHAII index cases and their affected relatives (five trios and one quartet) genome-wide SNP genotyping was performed using Illumina Human610-Quad BeadChips and GenomeStudio software. Approximately 40K tag SNPs were extracted using Plink. Analysis of linkage was performed using Merlin, specifying an autosomal dominant model with no phenocopies. Variants from exome sequencing in regions of the genome that were excluded (lod score < −2) were removed from further analysis, while those that supported linkage were prioritized for further evaluation. In kindreds showing potential recessive transmission of PHAII, SNP genotypes were examined for regions of homozygosity, and linkage was performed specifying an autosomal recessive model.

Sanger sequencing of KLHL3 and CUL3. PCR amplification and Sanger sequencing from genomic DNA was performed using standard methods. Primers were designed with Primer3. Variants identified by exome sequencing were verified. All exons and flanking intronic sequences of KLHL3 and CUL3 were sequenced from all PHAII index cases. Previously unidentified mutations were discovered and verified by independent amplification and sequencing. Co-segregation of mutations with disease was determined by sequencing in all available kindred members. CUL3 exon 9 and its flanking intronic sequence was sequenced in 150 unaffected unrelated controls, none of whom were found to harbour previously unidentified variants. It is noteworthy that because of lower or absent sequence coverage at or near intron–exon junctions, splice donor and acceptor mutations in CUL3 were suggested in two of the eleven PHAII exomes (SAMTools quality scores 96 and 75) but three branch site mutations were outside the exome sequence and one splice enhancer mutation was poorly covered (SAMTools quality score 3).

Genome-wide assessment of mutation burden. Genes show substantial variation in the prevalence of novel or rare protein-altering variants for biological reasons, including differences in gene size and variation in the proportion of bases that are under purifying selection, and for technical reasons, including difficulties in accurately mapping short sequence reads among closely related paralogues. These factors can limit the ability to directly identify disease loci by simply counting and ranking genes according to the absolute number of such variants, particularly for diseases with substantial locus or model heterogeneity. This gene-to-variant variation can be accounted for by use of control exome data. The prevalence of rare variation in each gene in case exomes was compared to the corresponding prevalence in a large set of control exomes with a Fisher’s exact test. Variants included in the analysis were protein-altering (missense, nonsense and splice site mutations) and high quality (≥8 independent reads and SAMTools quality score ≥100).

For a gene-wise test of rare variant burden in a genome with ~21,000 genes, correction for multiple testing suggests a threshold P value of ~2.4 × 10⁻⁶, anticipated to produce a false discovery rate (FDR) of one gene per twenty experiments. The FDR of the Fisher’s test was evaluated by Monte Carlo simulation, which confirmed an FDR of <1 gene per 20 experiments (Supplementary Table 1). The power to identify trait-related loci was estimated as a function of the number of variants detected in cases and the number of case exomes sequenced (Supplementary Table 2), and the test was applied comparing the eleven PHAII and 699 control exomes (Supplementary Table 3).

Orthologue and paralogue comparisons. Protein sequences of orthologues and paralogues were aligned with Clustal W. Crystal structures were examined with Cn3D. The locations of KLHL3 propeller mutations were compared to the crystal structure of human KLHL2 (PDB accession 2XN4), the closest human parologue (85% amino acid identity in the propeller). The location of the peptide encoded by CUL3 exon 9 was approximated by comparison to the crystal structure of human CUL1 (PDB accession 1LD1 and 1LDK).21

Splicing assay. A 3,782 bp segment of CUL3, extending from 287 bp proximal to exon 8 to 327 bp distal to exon 10, was amplified by PCR (Advantage 2 polymerase, Clontech) from genomic DNA of nine PHAII patients with different CUL3 mutations and one subject with wild-type CUL3 sequence. Products were cloned into the pCNA6.2/GW/D-TOPO mammalian expression vector (Invitrogen), and plasmids were purified (QIAprep, Qiagen) and sequenced. HEK293 cells were transfected independently with each plasmid using Lipofectamine 2000 (Invitrogen) and harvested ~24 h post-transfection. RNA was isolated using RNeasy with DNase on-column digestion (Qiagen). The spliced expression products were assessed by reverse transcription with oligo(dT) priming (Superscript III RT, Invitrogen) followed by PCR with vector-specific and CUL3-specific primers. Products were fractionated and visualized via agarose gel electrophoresis, and sequenced.

Immunofluorescence. Fresh frozen mouse kidney sections were fixed with ethanol at 4 °C for 30 min and acetone at −20 °C for 3 min, washed with PBS, and permeablized with 0.1% Triton X-100 (Sigma) at room temperature (22 °C) for 10 min. Sections were blocked with 10% donkey serum and 1% bovine serum albumin at room temperature for 1 h, incubated with primary antibodies at room temperature for 1 h or 4 °C overnight, washed four times with 1× PBS, incubated with secondary antibody at room temperature for 1 h, and washed four times with 1× PBS, with DAPI nuclear counterstain in the second wash. Slides were mounted with Mowiol (Polysciences) and 1% n-propyl gallate (Sigma) as an anti-fade agent.

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