Supporting Information

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SI Methods

Development of BHD Knockout Mouse Model. The BHD heterozygous knockout mice were generated as previously described (1). The BHD+/− mice, which carried the floxed allele, were crossed with β-actin-cre mice to delete the genomic region flanked by loxP sites. Deletion of exon 7 causes a frameshift and produces a premature termination codon in the beginning of exon 8, which should result in degradation of the mRNA by nonsense mediated decay (NMD). BHD deletion and inactivation was confirmed by qRT-PCR of BHD mRNA extracted from conditionally targeted kidneys from our BHD/KSP Cre mouse model previously described (1). We confirmed the absence of truncated BHD protein (FLCN) in BHD+/− mouse kidneys by Western blotting using anti-FLCN antibodies that react with the N-terminal portion of FLCN. All mice which were used in these experiments were housed in the NCI-Frederick animal facility in standard cages with food and water ad libidum, grouped by age, sex and strain according to the NCI-Frederick Animal Care and Use Committee guidelines. C57BL/6 mice were purchased from Charles River Laboratories. All other mouse strains were produced in-house. Animal care procedures followed National Cancer Institute (NCI)-Frederick Animal Care and Use Committee guidelines.

PCR-Based BHD Genotyping. Mouse genomic DNA was isolated from tails (weaned neonates), yolk sacs (E8.5 or later), and whole embryos (E7.5 or earlier). KOD Hot start DNA polymerase (Toyobo) was used for generating probes and routine PCR (PCR) genotyping. PCR genotyping was performed with three primer sets to amplify wild-type (178 bp PCR product), floxed (210 bp PCR product), and deleted (392 bp PCR product) BHD alleles: P1, 5′-GGTGTCTGGAGTGTCATTTGAGG-3′, which is complementary to the genomic sequence upstream of the 5′-loxP sequence; P2, 5′-ACAACCCCCAGCATCCAG-3′, which is complementary to the sequence downstream of 5′-loxP; and P3, 5′-CAGCTCCCTTACCCAGAC-3′, which is complementary to the sequence downstream of 3′-loxP.

PCR genotyping was performed on laser-capture microdissected embryo regions. For the LCMed samples, Crimson Taq polymerase (New England Biolabs) and different primers were sected embryo regions. For the LCMed samples, Crimson Taq polymerase (New England Biolabs) and different primers were

Quantitative Real Time-PCR. qRT-PCR of normal mouse tissue was performed using Mouse Major-Tissue Rapid-Scan containing single strand DNA from 24 major mouse organs (Origene). The primer sequences were: mouse-FLCN forward; 5′-TCCACCATGAGACAGCAGCAACGCA-3′, mouse-FLCN reverse; 5′-TGCACTGACCCACACACTTCTC-3′. For AKT expression in mouse kidney tissues, qRT-PCR was performed according to manufacturer’s instructions. Total RNA was isolated from frozen kidney tissues and tumors using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The following PCR primers to amplify β-actin and AKT 1 and 2 were generated by using Primer express software: β-actin forward; 5′- GGTGATGTCATGCGTCTTA-3′, β-actin reverse; 5′- CAGGCTCCCTCTACCCAC-3′, AKT1 reverse; 5′-GGACCCCTTAGAGACGTTGG-3′ (GenBank accession no. NM_009652.2); AKT2 forward: 5′-TGGGCTCCCTCTGTTTCCAG-3′, AKT2 reverse: 5′-CACCTCTTGGAGGCTATGG-3′ (GenBank accession no. NM_001110208.1). Three independent experiments were performed in triplicate using the β-actin gene as an internal control.

Whole Mount in Situ Hybridization. The embryos were collected from wild-type C57BL/6 mice intercrossed at different stages of gestation, fixed overnight in 4% PFA, and processed for whole mount in situ hybridization as previously described (2). The primers for the BHD probe were designed and synthesized from exons 5–11 of the BHD mRNA sequence (forward; 5′-GCCCTCAGGTGTGGTGAGCAGA-3′ and reverse; 5′-TCCACGACAACTACGACTTGAGGC-3′).

Histological and Immunohistochemical Analysis. Embryos were isolated at 5.5–6.5 dpf and fixed overnight in 4% PFA then embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Immunohistochemical analysis for DAB2 was performed as previously described (3). The primary antibody for DAB2 (BD Biosciences) was diluted 1:100. Primary antibody was replaced by normal mouse IgG antibody for the negative controls. The slides were read by at least three persons, including two pathologists (Drs. M.J. Merino and D.C. Haines).

Western Blotting and Antibodies. The aged mice were euthanized by CO2 asphyxiation, their kidneys were removed, cut into small pieces, snap frozen in liquid nitrogen, and stored at −80 °C for further analysis. For each mouse, one frozen kidney piece was homogenized in RIPA buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 1.0% Triton X-100, 0.5% deoxycholate, 0.1% SDS (SDS), 100 nM Calyculin A, and Complete Protease Inhibitor mixture (Roche)] with a Polytron homogenizer on ice followed by centrifugation at 16,000 × g for 30 min. Protein concentrations of cleared supernatants were measured with BCA Protein Assay Kit (Pierce Biotechnology, Inc.) and adjusted to 1.33 mg/mL, 4× SDS-sample buffer was added, and samples were boiled for 5 min to produce 1 mg/mL sample lysates. A total of 20 μg protein was loaded onto 4–20% or 4–15% SDS/PAGE gels. Immunoblotting was performed as previously described (1). Antibodies were diluted as follows: mouse monoclonal FLCN 1:1,000, β-actin 1:250, p-AKT (Thr308) 1:1,000, AKT1 1:1,000, AKT2 1:1,000, p-mTOR (Ser2448) 1:1,000, Raptor 1:1,000, p-mTOR(Ser2481) 1:1,000, Rictor 1:1,000, mTOR 1:1,000, p-S6K(Thr421/Ser424) 1:1,000, 1:250, Cyclin D1 1:1,000, p-FoxO1a 1:2,000, p-FOXO3a 1:500 [all antibodies except FLCN and β-actin (Biomedical Technologies, Inc.) are from Cell Signaling]. Secondary antibodies were diluted as follows: goat anti-mouse IgG-horseradish peroxidase, 1:10,000; goat anti-rabbit IgG-horseradish peroxidase, 1:10,000 (Vector laboratories). Antibody-protein complexes were detected using SuperSignal West pico chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturer’s instructions.

Tissue Genotyping by Southern Blotting. A 3′ external probe for Southern blot analysis of the tumors from BHD+/− mice and normal kidneys was generated by PCR with primers: forward; 5′-GACCCGACCACATGTCG-3′, reverse; 5′-GGACCCCTTACAGGTCGGTGG-3′ (GenBank accession no. NM_00110208.1). Three independent experiments were performed in triplicate using the β-actin gene as an internal control.
CAGGCTCAAGCAGTAGTGAGACCA-3'; and reverse; 5'-TGATGAATGCGACAGCAGCAG-3' (GenBank NT 096135.5). Nonradioactive Southern blotting was performed with DIG OMNI System for PCR Probes according to the manufacturer's protocol (Roche).

Endogenous FLCN Detection by Duolink System. Five-micrometers-thick frozen sections were prepared and mounted onto positively charged slides, fixed in methanol/acetone (1:1) at −20 °C for 10 min, dried and blocked with 10% normal goat serum. They were then rinsed with PBS, quenched in 0.5M ammonium chloride/0.1% BSA (BSA) (in PBS for 15 min at room temperature, washed with PBS, blocked with M.O.M. blocking system(Vector Laboratories) for 1 h at room temperature, and were incubated with combination of the primary anti-FLCN antibodies overnight at 4 °C. The primary antibodies were diluted as follows: FLCN mouse monoclonal antibody 1:200, FLCN rabbit polyclonal antibody 1:200, FL-342 FLCN antibody (Santa Cruz Biotechnology, Inc.), 1:200. PBS with 0.1% Tween-20 (PBS-T) was used for washing. Duolink in situ PLA was performed per manufacturer's instruction (OLink Biosciences). Briefly, after incubation with primary antibodies, we applied combinations of corresponding PLA probes (i.e., anti-rabbit PLUS, anti-mouse MINUS PLA probes) for 1 h at 37 °C. Subsequent hybridizations, ligation and detections using Duolink 100 Detection Kit 563 (OLink Biosciences) were performed. The kit 563 includes a Tye 563 fluorophore with excitation at 557 nm and emission at 563 nm and Hoechst 33422 nuclear dye. As negative control, normal mouse IgG antibody and normal rabbit IgG antibody were used at the same dilution. Images were taken using a LSM 710 confocal laser scanning microscope (Carl Zeiss).

Human Sample Preparation and MSD Analysis. Renal tumors were obtained from BHD patients surgically treated at the Urologic Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD (with patient permission under a National Institutes of Health IRB approved protocol #97-C-0147). All patients signed informed consent. Tumors were procured in the operating room to preserve the tissue intact with minimal effect of hypoxia. The tissues used in this study were snap frozen in liquid nitrogen from 4–11 min after they were removed from the body (Fig. S3D) and stored at −80 °C for further analysis. Tissues were lysed as described above. The protein concentrations were measured using the BCA method according to the manufacturer's instructions (Pierce Biotechnology, Inc.).

MSD (Meso Scale Discovery) 96-well multipot AKT signaling pathway [p-AKT (Ser473)/total GSK3β/p-S6K (Thr421/Ser424)] and p-S6R (Ser240/244) assays were carried out according to the manufacturer’s protocol. Briefly, plates were blocked (MSD blocking solution plus 3% BSA, as recommended by the manufacturer) for 1 h at room temperature with shaking and washed four times with Tris-buffered saline with 0.1% Tween-20. Ten to 20 micrograms of protein was added to the plate in triplicate wells and shaken for 1 h to 3 h at room temperature. Plates were washed as previously; then 25 µL detection antibody was added and incubated at room temperature for 1 h with shaking. Plates were washed four times with Tris-buffered saline with 0.1% Tween-20 as before, 150 µL read buffer was added, and the plates were analyzed on a SECTOR™ 6000 instrument (Meso Scale Discovery). The one or three additional spots in each well were coated with BSA. Both the phospho- and total signals were corrected for background (BSA spots) and for any effects of the lysis buffer.

Immunofluorescence Imaging of the AKT-mTOR Pathway. Five-micrometers-thick frozen sections were prepared and mounted onto positively charged slides, fixed in methanol/acetone (1:1) at −20 °C for 10 min, and blocked with 10% normal goat serum. They were then rinsed with PBS, quenched in 0.5M ammonium chloride/0.1% BSA (BSA) in PBS for 15 min at room temperature, washed with PBS, and incubated with the primary antibody in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% (vol/vol) Tween-20, 3% goat serum, and 0.1% (wt/vol) BSA at 4 °C overnight. Antibody dilutions were as follows: p-AKT 1:100, p-mTOR 1:100, p-S6R 1:100, and p-GSK3β 1:100. After three 10-min washes with TBST [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% (vol/vol) Tween-20] (TBST = Tris buffered saline with Tween-20), slides were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:500 dilution) and/or Alexa Fluor 594 goat anti-mouse IgG (1:500 dilution) (Invitrogen). After another three 10-min washes with TBST, slides were sealed with mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI, 1×, Vector Laboratories), and viewed with an LSM 710 confocal microscope system (Carl Zeiss).

Fig. S1. Endogenous BHD mRNA levels in mouse tissues and wild-type embryos. Quantitative RT-PCR was performed using Mouse Rapid-Scan Gene Expression Panels to determine BHD mRNA levels in mouse embryos and tissues. The mRNA levels are relative to the amount of BHD mRNA in the liver, which is defined as 1.
Fig. S2. Whole mount in situ hybridization with BHD mRNA probes showing wild-type mouse embryo development. At least five embryos were examined at each stage and all of the embryos showed similar patterns. (G', H', I', and J') Sections of the embryos shown in (G–J) were as indicated by the yellow lines. BHD mRNA was expressed consistently throughout embryogenesis. At E5.5, BHD expression was restricted to extraembryonic tissues (A), but by E6.5, BHD was expressed in both embryonic and extraembryonic tissues (B). At E7.5, a strong signal was observed in the neural ectoderm of the primitive streak region (arrowhead; D) and in the headfold (hf) (E) of the ectoderm; however, the signal was consistently weaker in the surrounding endoderm (C–F). Interestingly, the signal in heart (ht) was relatively weak (F and G with insert G' showing heart section). At E9.5, BHD was expressed almost ubiquitously, especially in the neural tube (H and H') and optic pit (I and I'). At E10.5, BHD was highly expressed in the branchial arch (ba), forelimb (fl), and hind limb (hl) (J), as well as in the somites (J').
Fig. S3. Phenotypic features of the renal tumors which were analyzed in this study. (A) Abdominal CT scan of BHD patient #2 (Fig. 6G) showing tumors in both kidneys (arrows). (B) Pathological phenotype of tumor #2 showing hybrid oncocytic features, the most common histology of renal tumor found in BHD patients (4). (C) Tumor #3 contained predominantly chromophobe renal carcinoma cells. Magnification: ×20. (Scale bar, 10 μm.) (D) All specimens used in this study were procured in the operating room. The tissues were snap frozen from 4–11 min after they were removed from the body to preserve the tissue intact with minimal effect of hypoxia.

<table>
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<th>Histology</th>
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Fig. S4. FLCN interaction with the PI3K–AKT–mTOR pathway.
Targeting strategy of BHD deletion in the mouse. A neomycin resistance (Neo^r^) cassette, flanked by Frt and loxP sequences, was inserted into intron 6 of BHD for positive selection, and the thymidine kinase gene was included for negative selection. A second loxP sequence was inserted into intron7 (A). BHD wild type allele (B), BHD targeted allele (C). The Neo cassette was excised by crossing with β-actin-Flp mice (D). Then the mice which carried the floxed allele were crossed with β-actin-cre mice to delete the genomic region flanked with loxp sites (E). Deletion of exon 7 causes a frameshift and produces a premature termination codon in the beginning of exon 8, which should result in degradation of the mRNA by nonsense mediated decay (NMD) (F).

Fig. S5.