Folliculin-interacting proteins Fnip1 and Fnip2 play critical roles in kidney tumor suppression in cooperation with Flcn

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Folliculin (FLCN)-interacting proteins 1 and 2 (FNIP1, FNIP2) are homologous binding partners of FLCN, a tumor suppressor for kidney cancer. Recent studies have revealed potential functions for FLCN in kidney; however, kidney-specific functions for Fnip1 and Fnip2 are unknown. Here we demonstrate that Fnip1 and Fnip2 play critical roles in kidney tumor suppression in cooperation with Flcn. We observed no detectable phenotype in Fnip2 knockout mice, whereas Fnip1 deficiency produced phenotypes similar to those seen in Flcn-deficient mice in multiple organs, but not in kidneys. We found that absolute Fnip2 mRNA copy number was low relative to Fnip1 in organs that showed phenotypes under Fnip1 deficiency but was comparable to Fnip1 mRNA copy number in mouse kidney. Strikingly, kidney-targeted Fnip1/Fnip2 double inactivation produced enlarged polycystic kidneys, as was previously reported in Flcn-deficient kidneys. Kidney-specific Flcn inactivation did not further augment kidney size or cystic histology of Fnip1/Fnip2 double-deficient kidneys, suggesting pathways dysregulated in Flcn-deficient kidneys and Fnip1/Fnip2 double-deficient kidneys are convergent. Heterozygous Fnip1/homozygous Fnip2 double-knockout mice developed kidney cancer at 24 mo of age, analogous to the heterozygous Flcn knockout mouse model, further supporting the concept that Fnip1 and Fnip2 are essential for the tumor-suppressive function of Flcn and that kidney tumorigenesis in human Birt–Hogg–Dubé syndrome may be triggered by loss of interactions among Flcn, Fnip1, and Fnip2. Our findings uncover important roles for Fnip1 and Fnip2 in kidney tumor suppression and may provide molecular targets for the development of novel therapeutic strategies for kidney cancer.

Folliculin | FNIP1 | FNIP2 | Birt–Hogg–Dubé syndrome | kidney tumor

Germline alteration of the folliculin (FLCN) gene, a novel tumor suppressor, is responsible for Birt–Hogg–Dubé (BHD) syndrome, an inherited kidney cancer syndrome characterized by cutaneous fibrofolliculomas, pulmonary cysts, and an increased risk for the development of kidney cancer (1–4). Genetic studies using Flcn knockout mice have defined important roles for Flcn in metabolism. Kidney-targeted Flcn knockout mice developed enlarged polycystic kidneys with elevated mechanistic target of rapamycin complex 1 (mTORC1) activity (5) and increased mitochondrial biogenesis through up-regulated peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Ppargc1a) (6). Muscle-targeted Flcn knockout mice displayed both red-colored muscle with increased mitochondrial biogenesis caused by elevated Ppargc1, and cardiac hypertrophy with up-regulated mTORC1, which were ameliorated by Ppargc1a inactivation, suggesting that Flcn might serve as a critical link connecting mTORC1 with Ppargc1a-driven mitochondrial biogenesis (5–7). Importantly, mice heterozygous for Flcn inactivation develop renal tumors at 24 mo of age with increased mTORC1/2 activity and Ppargc1a expression, which mimics the human BHD tumor phenotype (6, 8–10), suggesting potential therapeutic targets in metabolic pathways for BHD-associated kidney cancer.

The first Flcn interacting protein FNIP1 was identified through protein–protein interaction studies of the FLCN protein (11). FNIP1 binds to the C terminus of FLCN and to AMP-activated protein kinase (AMPK) (11), a critical molecule for energy sensing, further underscoring a central role for the FLCN/FNIP1 pathway in cellular metabolism. A second folliculin-interacting protein FNIP2 was discovered through bioinformatics searches for sequences similar to FNIP1 (12, 13). Similar to FNIP1, FNIP2 was found to bind to the C terminus of FLCN and to AMPK (12), suggesting a potential functional redundancy with FNIP1. Recent studies with Fnip1 knockout mouse models have demonstrated that Fnip1 is required for B-cell development.

Significance

The role of FLCN as a tumor suppressor in kidney cancer has been well documented, whereas the functional roles of folliculin (FLCN)-interacting proteins 1 and 2 (FNIP1 and FNIP2) in kidney are unknown. In this study, we demonstrate that double inactivation of Fnip1 and Fnip2 leads to enlarged polycystic kidneys or kidney cancer, which mimics the phenotypes seen in Flcn-deficient kidneys and underscores the significance of Fnip1 and Fnip2 in kidney tumor suppression. Moreover, we found that Fnip1/Fnip2 mRNA ratios differ among organs, which may reflect tissue-specific roles for each Fnip. Our findings define Fnip1 and Fnip2 as critical components of the Flcn complex that are essential for its tumor suppressive function and will aid in the development of novel therapeutic strategies for kidney cancer.

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Interestingly, a Flcn knockout mouse model using the tamoxifen-inducible ER (mutated form of the ligand-binding domain of the estrogen receptor)-Cre system also displayed defects in B-cell development (14), suggesting Fnip1 knockout mice might develop phenotypes similar to those that develop as a consequence of Flcn deficiency.

Furthermore, FLCN has been shown to have a variety of functions that might potentially link AMPK, mTOR, and Ppargc1a with other important pathways. Crystallographic studies have shown that the C terminus of FLCN may be distantly related to Differentially Expressed in Normal Cells and Neoplasia (DENN) domain proteins and may possess guanine nucleotide exchange factor activity toward RASB35 (16). FLCN modulates TFE3 localization (17), which may play an important role in the exit of stem cells from pluripotency (18), and interacts with other signaling pathways including the von Hippel-Lindau–hoxi oxidase inducible factor–vascular endothelial growth factor axis (19–21), the TGFB-beta pathway (22, 23), Rho A signaling (24, 25), cell cycle regulation (26, 27), Rag-mediated amino acid sensing (28, 29), and autophagy (30, 31). These findings underscore FLCN as an important molecule, inactivation of which affects multiple pathways.

To clarify the function of FLCN-interacting proteins Fnip1 and Fnip2, we inactivated Fnip1 and/or Fnip2 in mouse kidneys, muscle, and heart and investigated the effect on the mTOR pathway and mitochondrial metabolism. The absolute mRNA copy number of Fnip1 and Fnip2 was measured using droplet digital PCR (ddPCR) technology. To evaluate functional synergy of Fnip1 and Fnip2 with Flcn, we also inactivated Flcn, Fnip1, and Fnip2 simultaneously in mouse kidneys. Finally, we searched for latent tumor development in Fnip1 and Fnip2 knockout mice.

Results

Neither Kidney-Targeted Fnip1 nor Fnip2 Knockout Mice Develop a Kidney Phenotype. To investigate Fnip1 function in mouse kidney, we crossed mice carrying loxP-flanked Fnip1 alleles (floxed, f) (14) with cadherin 16 (CDH16)-Cre transgenic mice, which express Cre recombinase driven by the CDH16 promoter, thereby deleting Fnip1 gene sequences specifically in kidney. We observed no significant phenotype in the Fnip1-deficient kidneys except occasional tiny cysts (Fig. 1A). Therefore, we decided to analyze Fnip2 function in kidney by generating a Fnip2 conditional mouse carrying loxP-flanked Fnip2 alleles (floxed, f) (15) and crossbreeding with CDH16-Cre transgenic mice. However, kidney-targeted Fnip2 inactivation also did not cause any phenotype in mouse kidney (Fig. 1E). Indeed, we could not find any phenotype in whole-body Fnip2 knockout mice that affected life span.

The Relative Expression Levels of Fnip1 and Fnip2 Differ from Organ to Organ. Previously, we reported that Fnip1 knockout mice showed B-cell developmental defects, which were also observed in Flcn knockout mice using the tamoxifen-inducible ER-Cre system (14), suggesting Fnip1 knockout mice might show phenotypes similar to those resulting from Flcn deficiency. In addition to the B-cell phenotype, we found similar Flcn-deficient and Fnip1-deficient phenotypes in skeletal muscle and heart. Muscle-targeted Fnip1 knockout mice showed red-colored muscle with increased mitochondrial biogenesis (myoglobin and cox4 readouts: Fig. 2A and B), as well as cardiac hypertrophy with elevated mTORC1 activity (Fig. 2C–F), which we had previously observed in muscle-targeted Flcn knockout mice (6, 32). Sequence similarity between Fnip1 and Fnip2 and the shared interaction of Fnip1 and Fnip2 with Flcn and AMPK (12) implied that Fnip1 and Fnip2 might be functionally redundant. In support of this, we observed that expression of either Fnip1 or Fnip2 in Fnip1/Fnip2 null mouse embryonic fibroblasts (MEFs) suppressed Ppargc1a mRNA and ATP production (Fig. 2 G–I). Because of the potential functional redundancies between Fnip1 and Fnip2, we postulated that Fnip1 and Fnip2 expression might differ from organ to organ and that this variable expression may determine the specific roles for Fnip1 and Fnip2 in those tissues. The recent technology of ddPCR enabled us to measure absolute mRNA copy number. Using this technology, we compared the absolute copy number of Fnip1 and Fnip2 mRNA in wild-type mouse tissues. Interestingly, we observed dominant expression of Fnip1 in heart, skeletal muscle, and bone marrow, the tissues in which we observed phenotypes in Fnip1 knockout mice, whereas there was no significant difference between Fnip1 and Fnip2 mRNA copy number in kidney (Fig. 2J), in agreement with the absence of kidney phenotypes in Fnip1 and Fnip2 knockout mice. These data support the idea that in kidney, the Fnip2 expression level, which is commensurate with that of Fnip1, might maintain Fnip function in Fnip1-deficient kidneys, and therefore, double inactivation of Fnip1 and Fnip2 would be necessary to develop a kidney-specific phenotype.

Kidney-Targeted Fnip1/Fnip2 Double-Knockout Mice Develop Enlarged Polycystic Kidneys. Strikingly, kidney-specific inactivation of both Fnip1 and Fnip2 resulted in enlarged kidneys (Fig. 3A). MRI imaging revealed multiple round-shaped structures in the Fnip1/Fnip2 double-deficient kidneys (Fig. 3B), which were confirmed by H&E staining to be polycystic lesions (Fig. 3C) displaying hyperplastic cells that protruded into the cystic lumen (Fig. 3D). Kidney-specific Fnip1/Fnip2 double-knockout mice showed a significantly increased kidney/body weight ratio (n = 12; percentage kidney/body weight ratio mean = 11.04%; P < 0.001) (Fig. 3E). The survival time of kidney-targeted Fnip1/Fnip2 double-knockout mice was about 3 wk (n = 14; median survival = 20 d; P < 0.0001) (Fig. 3F). The enlarged polycystic kidneys of kidney-targeted Fnip1/Fnip2 double-deficient mice were reminiscent of the kidney phenotype observed in Flcn-deficient kidneys (5), underscoring the phenotypic similarities between these two genotypes.

Fnip1/Fnip2 Double-Deficient Kidneys Are Idential to Flcn-Deficient Kidneys. Previously we observed increased mTOR activity (5) and Ppargc1a-driven mitochondrial biogenesis in Flcn-deficient kidneys (6). In fact, Fnip1/Fnip2 double-deficient kidneys showed increased protein expression of Ppargc1a and signaling molecules in the mTOR pathway, including the downstream target of mTORC1, phospho-Ulk1 at Ser757, that suppresses autophagy, which was confirmed by the accumulation of sequestosome-1 (SQSTM1)/p62 (Fig. 4 A and B). Increased respiratory capacity (n = 4 each; P < 0.001) (Fig. 4C) and increased mitochondrial surface area (13 cells each; P < 0.001) (Fig. 4D) were also observed in the Fnip1/Fnip2 double-deficient kidneys. These data further support the concept that double inactivation of Fnip1/Fnip2 in kidney cells mirrors the same phenotype as that associated with kidney-targeted Flcn inactivation. We next asked whether the enlarged polycystic kidney phenotype in kidney-targeted Fnip1/Fnip2 double-knockout mice developed through the same pathway that had produced the identical phenotype in kidney-specific Flcn knockout mice. To answer this question, we crossedbred kidney-specific Fnip1/Fnip2 double-knockout mice with kidney-specific Flcn knockout mice to see whether Flcn inactivation might have a synergistic effect on Fnip1/Fnip2 doubly inactivated kidneys. In fact, Flcn inactivation did not enhance the size (n = 8; P = 0.554) (Fig. 4E) or alter the histology (Fig. 4F) of Fnip1/Fnip2 double-deficient kidneys, suggesting that the enlarged polycystic kidney phenotypes of kidney-specific Fnip1/Fnip2 double-knockout mice and kidney-specific Flcn knockout mice developed through the same pathway.
Neither kidney-targeted Fnip1 nor Fnip2 knockout mice showed a kidney phenotype. (A) Conditional Fnip1 knockout mice were crossed with CDH16-Cre transgenic mice. Inactivation of Fnip1 mRNA was confirmed by real-time PCR. n = 6 each at 3 wk of age. Mean ± SD. Student t test (Left). The size of the Fnip1-deficient kidney was not significantly different from that of the control kidney. n = 11 each at 3 wk of age. Mean ± SD. Student t test (Middle). Representative H&E staining of 3-wk old control and Fnip1-deficient kidneys did not show differences except for infrequent tiny cysts (Right). (B) Fnip2 gene-targeting vector was constructed by recombineering methodology using homologous recombination. A neomycin resistance (Neo) cassette flanked by Frt (bar) and loxP (triangle) sequences was inserted into intron 11 for positive selection, and the thymidine kinase gene was included for negative selection. A second loxP sequence was inserted into intron 13. Correctly targeted embryonic stem cells were identified by Southern blot analysis and injected into blastocysts to produce chimeras. Backcrossing to C57BL/6 mice produced heterozygous F1 offspring with germine transmission of the Fnip2 floxed (flox)-Neo allele. The Neo cassette flanked by Frt sites was excised in vivo by crossing with mice expressing the Flp recombinase transgene under the β-actin promoter. To produce the Fnip2 deleted (delt) allele, Fnip2 flox-Neo mice were crossed with mice expressing the Cre recombinase transgene under the ubiquitous β-actin promoter. Deletion of exon 12 and 13 resulted in a frameshift and premature termination codon in exon 14, which was predicted to cause mRNA degradation by the nonsense-mediated decay mRNA surveillance system. (C) The targeted embryonic stem cells were screened by Southern blotting of BamH1- and EcoRV-digested DNA, using two different external probes located outside the targeting sequence, as shown in B. (D) PCR-based genotyping was performed using DNA extracted from mouse tails for routine monitoring of inheritance in offspring. Locations of PCR primers are indicated by arrows. (E) Kidney-specific inactivation of Fnip2 was achieved by crossing with CDH16-Cre transgenic mice. Inactivation of Fnip2 mRNA was confirmed by real-time PCR. n = 6 each at 3 wk of age. Mean ± SD. Student t test (Left). Size of Fnip2-deficient kidney was not significantly different from that of control kidney. n = 6 each at 3 wk of age. Mean ± SD. Student t test (Middle). Representative H&E staining of 3-wk old control and Fnip2-deficient kidneys did not show any difference in histology (Right).
and immunostaining (Fig. 5E), further supporting the idea that these tumors might develop through the same pathway as tumors that develop in the heterozygous Flcn knockout mouse model.

**Discussion**

Here we report kidney tumor-suppressive roles for Fnip1 and Fnip2 in cooperation with Flcn. *Fnip1/Fnip2* double inactivation targeted to mouse kidney resulted in an enlarged multicystic kidney phenotype shortly after birth, which was identical to the phenotype observed in *Flcn*-deficient kidneys (5). The ratio of absolute *Fnip1* to *Fnip2* mRNA copy number was high in the organs that demonstrated a phenotype after *Fnip1* inactivation, whereas in kidney where no *Fnip1*-deficient or *Fnip2*-deficient phenotype was observed, the absolute *Fnip2* mRNA copy number was comparable to that of *Fnip1*. Therefore, these data suggest that the ratio of absolute *Fnip1* to *Fnip2* mRNA copy number may determine the function of each *Fnip* in a particular organ. Moreover, whole-body heterozygous *Fnip1/homozygous Fnip2* double-knockout mice developed kidney tumors at 24 mo of age, implying that loss of interaction between Flcn, Fnip1, and Fnip2 may trigger kidney cancer development.

According to protein sequence, FNIP1 and FNIP2 show 49% identity and 74% similarity (12), suggesting possible functional redundancy. Double homozygous inactivation of *Fnip1* and *Fnip2* specifically in mouse kidney resulted in an enlarged polycystic kidney phenotype. However, expression of one allele of either *Fnip1* or *Fnip2* in kidney-targeted *Fnip1/Fnip2* knockout mice was sufficient to rescue this phenotype, suggesting *Fnip1* and *Fnip2* may have functional redundancy. Because worms and flies have only one *Fnip* protein (12), it is possible that a second *Fnip* with overlapping functions evolved from the primary *Fnip* to ensure redundancy and conserve its critical role in regulating kidney cell proliferation through its interaction with Flcn.

In contrast, the differences between FNIP1 and FNIP2 amino acid sequences imply potentially distinct functions for each FNIP. Therefore, the nonoverlapped functions of the FNIPs, as well as additional overlapping functions, will need to be elucidated in future experiments.

Crystallographic studies revealed that the C terminus of FLCN shows distant homology to DENN domain proteins, a family of GDP–GTP exchange factors that activate Rab GTPases involved in membrane trafficking in eukaryotes (16). Notably, a subsequent bioinformatics study reported that FNIP1 and FNIP2 also have novel DENN modules (33), raising the possibility that complex assembly of FLCN–FNIP1 or FLCN–FNIP2 might form a noncanonical DENN module critical for GDP–GTP exchange that would suffer functional arrest if any of the components were absent. Disruption of a noncanonical DENN module, whose conformation may be controlled by Flcn/Fnip1 or Flcn/Fnip2 interactions and may be essential for regulation of proper kidney cell proliferation rates, might result in development of the polycystic kidney phenotype in our kidney-targeted in vivo models.
After **FLCN** was identified as a two-hit tumor suppressor gene for BHD-associated chromophobe, hybrid oncocytc, and clear cell kidney cancers, a search for **FLCN** mutations in a broad spectrum of sporadic kidney tumors was conducted, but genetic analyses of these samples only rarely identified mutations in the **FLCN** gene (34, 35). Somatic **FNIP1** or **FNIP2** mutations have been detected in several cancers by whole-exome sequencing as part of The Cancer Genome Atlas (TCGA) project. Sequencing efforts by TCGA project detected infrequent **FNIP1/FNIP2** mutations in urologic cancers, including clear cell renal carcinomas (7/424; 1.7%) and bladder cancer (7/130; 5.4%), and in all but one of the urologic cancer samples, the tumors had a single mutation of either **FNIP1** or **FNIP2** (36). In comparison, uterine corpus endometrioid cancer displayed the highest percentage of tumors with **FNIP1** and **FNIP2** alterations (17/240; 7.1%), of which 5 (2.1%) had mutations in both genes (37). Infrequent somatic mutations of **FLCN**, **FNIP1**, and **FNIP2** genes in sporadic kidney tumors indicate that genetic alteration of these genes is not the direct cause of sporadic kidney tumorigenesis, but rather, that the status of the **FLCN/FNIP1** or **FLCN/FNIP2** interactions might be critical for sporadic kidney tumor suppression. Protein–protein interaction studies of sporadic kidney cancers would be important to elucidate the functional status of the **FLCN/FNIP1**/ **FNIP2** complex in sporadic kidney cancer, especially in sporadic chromophobe renal cell carcinoma and oncocytyoma, which are the most frequent histologic subtypes observed in human BHD syndrome (Fig. S1).

The findings of this study, which characterize the phenotype of **Fnip1**, **Fnip2**, and **Fnip1/Fnip2** knockout mouse models in multiple organs, further our understanding of the Fcn tumor suppressor pathway and underscore important roles for Fcn/Fnip1/Fnip2 interactions in inhibiting kidney cancer development. These data may lead to the development of novel diagnostics and therapeutics for kidney cancer that target the **FLCN-FNIP** pathway.

**Materials and Methods**

**Animals.** Mice carrying **Flcn** alleles and **Fnip1** alleles flanked by loxP sites (floxed, f) and mice carrying a **Fnip1** deleted (d) allele were generated as previously described (5, 14). Mice carrying **Fnip2** alleles flanked by loxP sites (floxed, f) and mice carrying a **Fnip2** deleted (d) allele were generated using the same strategy (5, 14). Briefly, the **Fnip2** targeting vector was generated by inserting a neomycin resistance cassette flanked by Frt and loxP sequences into intron 11 of **Fnip2** and inserting a second loxP sequence into intron 13. Deletion of exon 12 and exon 13 in the **Fnip2** allele resulted in a reading frameshift and premature termination codon in exon 14. **Cdhn16**-Cre transgenic mice, which express Cre recombinase under the cadherin 16 (**Cdhn16**) promoter specifically in adult renal tubules and developing genitourinary tract (38), were crossed with mice carrying floxed (f) alleles of **Flcn**, **Fnip1**, and **Fnip2** to inactivate those genes. Because we did not observe any phenotypic difference between **Fnip1**<sup>ff</sup>, **Fnip2**<sup>ff</sup>, **Cdhn16-Cre** and **Fnip1<sup>d</sup>, Cdhn16-Cre** mice, we used these two genotypes interchangeably throughout these studies. Muscle-targeted inactivation of **Fnip1** was done by crossing **Fnip1<sup>d</sup>, Cdhn16-Cre** mice and **Ckmt-Cre** transgenic mice (Jackson Laboratories). Mice were housed in National Cancer Institute animal facilities and killed by CO<sub>2</sub> asphyxiation for analyses according to the National Cancer Institute–Frederick Animal Care and Use Committee guidelines. Animal care

(C) H&E staining shows enlarged polycystic kidneys in 3-wk-old **Fnip1<sup>d</sup>, Cdhn16-Cre** mouse. (Scale bars: 500 μm.) (D) H&E staining reveals detailed histology of kidneys from 3-wk-old kidneys targeted **Fnip1/Fnip2** double-knockout mice displaying hyperplastic cells protruding into the lumen (arrow) within the medulla (Upper). Normal glomeruli (G) and proximal renal tubules (P) were observed in the cortex (Lower). (Scale bars: 50 μm and 20 μm.) (E) Kidney-specific **Fnip1<sup>d</sup>, Cdhn16-Cre** double-knockout mice show an increased kidney/body weight ratio. Homozygous **Fnip1<sup>ff</sup>, Cdhn16-Cre** double-knockout mice show a slightly increased kidney/body weight ratio as a result of occasionally observed tiny cysts. Mean ± SD. Two-sided Student t test. Three weeks of age. (F) Survival curve of kidney-specific **Fnip1<sup>d</sup>, Cdhn16-Cre** double-knockout mice. Proportion surviving ± SD. Log rank test. n = 14 each at 3 wk of age.

Fig. 3. Kidney-targeted **Fnip1/Fnip2** double-knockout mice develop enlarged polycystic kidneys. (A) **Fnip1** and **Fnip2** alleles were deleted specifically in kidney using **Cdhn16-Cre** transgenic mice. Double inactivation of **Fnip1** and **Fnip2** targeted to the kidney resulted in enlarged kidneys relative to the controls (3 wk of age). (B) T2 weighted images (T2WI) of MRI show multiple round-shaped structures in **Fnip1/Fnip2** double-knockout kidneys at 3 wk of age (Left). Striations of medulla and renal pelvis are seen (Right).
Southern Blot Analysis of Embryonic Stem Cells and PCR Genotyping of Fnip2 Knockout Mice. KOD Hot DNA polymerase (Novagen) was used for generating probes and routine PCR genotyping. A 5′-external probe for Southern blot analysis of targeted embryonic stem cells was generated by PCR with the following primers: forward, 5′-GAACAAGGAGGGAAATCCA-3′; reverse, 5′-GCAAAGGCTGAAACCCTGCA-3′. A 3′-external probe was also generated by PCR with the following primers: forward, 5′-TACGCAAAACAA- TAGGCTCA-3′; reverse, 5′-TCAGCACTAGCCTGGGGARCA-3′. Nuclei were isolated from tail samples of mice using DirectPCR Reagent (Viagen Biotech, Inc.) according to manufacturer’s protocols. Nonradioactive Southern blotting was performed with DIG OMNI System for PCR probes (Roche Molecular Biochemicals) according to the manufacturer’s protocol. PCR genotyping was performed with three primer sets to amplify wild-type (146-bp PCR product), floxed (238-bp PCR product), and deleted (457-bp PCR product) Fnip2 alleles: P1, 5′-ATGGTCTAGGAGAGCCATTG-3′; P2, 5′-AGGACAGAAGGGACGTGCTAG-3′; and P3, 5′-AGTGTCAGACTTCCTTCTCGTG-3′. We confirmed that the CDH16-Cre transgenes had no detectable effect on mouse phenotypes.

dPCR and Real-Time PCR. mRNA was extracted from bone marrow, heart, kidney, and quadriceps of C57BL/6, using TRIzol (Invitrogen), and 500 ng mRNA was transcribed into cDNA, using SuperScript III reverse transcriptase (Invitrogen) with 10 μL scale. A microtiter of cDNA was used to make droplets using the QX100 ddPCR system (Bio-Rad), and droplets were amplified and analyzed with the Taqman assays for Fnip1 (Mm00620486_m1) labeled with FAM and for Fnip2 (Mm01220192_m1) labeled with VIC. Real-time PCR was done as previously described (6). Primer sequences are as follows: mouse Fnip1-forward, 5′-cacaggttagaagttggctgg-3′; mouse Fnip1-reverse, 5′-gcggagagccctagtgcttgg-3′; mouse Fnip2-forward, 5′-cggagaggggcttagggggtg-3′; mouse Fnip2-reverse, 5′-cggagaggggcttagggggtg-3′; mouse Pparcg1-forward, 5′-atgacctgctaaacaccaacacac-3′; mouse Pparcg1-reverse, 5′-cttaggcattggtgcgttggttt-3′; mouse β-actin-forward, 5′-gacatggagaagatctggca-3′; mouse β-actin-reverse, 5′-gtgtccaaagaccagttggtg-3′; and mouse β-actin-forward, 5′-gtgtccaaagaccagttggtg-3′. We confirmed that the CDH16-Cre transgenes had no detectable effect on mouse phenotypes.

MRI Imaging. T2-weighted images were obtained using a fast spin echo sequence (rapid acquisition of relaxation enhancement) with an echo time of 13 ms and a repetition time of 2,500 ms by a 7 T MRI scanner controlled with ParaVision 5.0 (Bruker BioSpin MRI GmbH).

Western Blotting, Immunofluorescence Staining, and Antibodies. Frozen kidney samples were homogenized in RIPA buffer (20 mM Tris HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 0.5% deoxycholate, 0.1% SDS) supplemented with Complete protease inhibitor mixture and PansStop phosphatase inhibitor mixture (Roche). For immunoblotting, 20 μg protein was loaded in each well. Immunofluorescence staining of kidney samples was done as previously reported (5, 9). Antibodies used for Western blotting included phospho-mTOR (Ser2448), phospho-mTOR (Ser2481), total mTOR, total AKT, phospho-AKT (Ser473), phospho-AKT (Thr308), phospho-S6K, phospho-p110α (Ser380, 381), and phospho-p70 (Thr38, 42). Antibodies used for Western blotting were shown to be specific for their respective proteins.

Immunofluorescence shows increased staining of p-mTOR (S2448) and pS6R (S240/244) in Fnip1/Fnip2 double-deficient kidney relative to control kidney. Nuclei were stained with DAPI (Blue). Representative of three mice at 3 wk of age. (C) Respiratory capacity of isolated mitochondria is increased in Fnip1/Fnip2 double-deficient kidney relative to control kidney. State 3 respiration of complex I and complex II, and complex IV-dependent respiration were measured by Seahorse XF96 analyzer. Mean ± SD. Student t test (two-sided). (D) Electron microscope images show increased mitochondrial mass in Fnip1/Fnip2 double-deficient kidney compared with control kidney. Arrows indicate mitochondria. (Scale bars: 10 μm and 500 nm.) Percentage of mitochondrial area per cell was quantified for the indicated genotypes. Thirteen cells were evaluated for each genotype. Mean ± SD. Student t test (two-sided). Three weeks of age. (E) The kidney/ body weight ratios of 3-wk-old mice with Flcn-deficient, Fnip1/Fnip2 double-deficient, and Flcn/Fnip1/Fnip2 triple-deficient mice show no significant differences. Mean ± SD, n = 8 each. Student t test (two-sided). (F) The histologies of kidneys from 3-wk-old Flcn-deficient, Fnip1/Fnip2 double-deficient, and Flcn/Fnip1/Fnip2 triple-deficient mice show no significant differences. Arrows indicate hyperplastic cells protruding into the cyst lumens that were observed in all of the genotypes. (Scale bars: 1 mm, 50 μm, and 20 μm.)
Mouse kidney samples were immediately immersed in 4% (vol/vol) formaldehyde/2% (vol/vol) glutaraldehyde (ElectroM Magnetic Sciences)/PBS. Small blocks were then cut, osmicated, and dehydrated before embedding. The blocks were sectioned and observed in a Hitachi H7600 (Tokyo, Japan) transmission electron microscope equipped with an XR418 CCD camera (Advanced Microscopy Techniques Corporation). Percentage mitochondrial area was analyzed with Image J (National Institutes of Health). The ratio of mitochondrial area to cell area was measured in 13 cells and represented as means and 95% confidence intervals.

Respiratory Capacity of Isolated Mitochondria from Kidney Tissue. For respiratory capacity measurements of isolated mitochondria from kidney, an XF96 V3 PET plate (Seahorse Bioscience) was coated overnight with 1:150,000 polydimethylsiloxane solution/assay buffer (137 mM KCl, 2 mM KH2PO4, 2.5 mM MgCl2, 20 mM Hepes, 0.5 mM EGTA, 0.2% fatty acid-free BSA). Mitochondria isolated from kidney using the standard Nagarse method was attached to the plate bottom at 936 g for 10 min. The plate was warmed at 37 °C for 10 min and transferred to the Seahorse XF96 analyzer. State III respiration (maximum ADP-stimulated oxygen consumption ratio under sufficient substrate for mitochondrial complex) of complex I was measured with 16 μM mitochondria immediately after the addition of 5 mM glutamate, 5 mM malate, and 0.5 mM adenosine diphosphate. State III respiration of complex II was measured with 8 μM mitochondria immediately after the addition of 5 mM succinate, 0.28 mM fumarate, and 0.5 mM adenosine diphosphate. Complex IV-dependent respiration was measured with 2 μM mitochondria immediately after the addition of 0.5 mM tetrathylphosphorylenediamine and 3 mM ascorbic acid.

Table 1. Neonates from matings of \textit{Fnip1}^{−/−} \textit{Fnip2}^{−/−} males and females

| Genotype                        | Neatones | Percentage neonates with genotype |
|---------------------------------|----------|-----------------------------------|
| \textit{Fnip1}^{−/−} \textit{Fnip2}^{−/−} | 47       | 36                                |
| \textit{Fnip1}^{−/−} \textit{Fnip2}^{+/−} | 85       | 64                                |
| \textit{Fnip1}^{−/−} \textit{Fnip2}^{−/−} | 0        | 0                                 |
| Total                           | 132      | 100                               |

\textit{Fnip1}^{−/−} \textit{Fnip2}^{−/−} males and \textit{Fnip1}^{−/−} \textit{Fnip2}^{−/−} females were mated, and 132 neonates were obtained from 53 litters. No \textit{Fnip1}^{−/−} \textit{Fnip2}^{−/−} neonates were observed.

Electron Microscopy. Mouse kidney samples were immediately immersed in 4% (vol/vol) formaldehyde/2% (vol/vol) glutaraldehyde (ElectroM Magnetic Sciences)/PBS. Small blocks were then cut, osmicated, and dehydrated before embedding. The blocks were sectioned and observed in a Hitachi H7600 (Tokyo, Japan) transmission electron microscope equipped with an XR418 CCD camera (Advanced Microscopy Techniques Corporation). Percentage mitochondrial area was analyzed with Image J (National Institutes of Health). The ratio of mitochondrial area to cell area was measured in 13 cells and represented as means and 95% confidence intervals.

Respiratory Capacity of Isolated Mitochondria from Kidney Tissue. For respiratory capacity measurements of isolated mitochondria from kidney, an XF96 V3 PET plate (Seahorse Bioscience) was coated overnight with 1:150,000 polydimethylsiloxane solution/assay buffer (137 mM KCl, 2 mM KH2PO4, 2.5 mM MgCl2, 20 mM Hepes, 0.5 mM EGTA, 0.2% fatty acid-free BSA). Mitochondria isolated from kidney using the standard Nagarse method was attached to the plate bottom at 936 g for 10 min. The plate was warmed at 37 °C for 10 min and transferred to the Seahorse XF96 analyzer. State III respiration (maximum ADP-stimulated oxygen consumption ratio under sufficient substrate for mitochondrial complex) of complex I was measured with 16 μM mitochondria immediately after the addition of 5 mM glutamate, 5 mM malate, and 0.5 mM adenosine diphosphate. State III respiration of complex II was measured with 8 μM mitochondria immediately after the addition of 5 mM succinate, 0.28 mM fumarate, and 0.5 mM adenosine diphosphate. Complex IV-dependent respiration was measured with 2 μM mitochondria immediately after the addition of 0.5 mM tetrathylphosphorylenediamine and 3 mM ascorbic acid.

(8) Kidney tumor-free survival demonstrates that heterozygous \textit{Fnip1} homozygous \textit{Fnip2} double-knockout mice developed renal tumors at a median age of 796 d. Neither heterozygous \textit{Fnip1} knockout mice nor homozygous \textit{Fnip2} knockout mice developed kidney cancer. Kidney tumor-free survival ± SD. Log rank test: n = 25, 15, and 42 for \textit{Fnip1}^{+/−}, \textit{Fnip2}^{−/−}, and \textit{Fnip1}^{−/−} \textit{Fnip2}^{−/−}, respectively. (C) H&E staining shows kidney tumor development in heterozygous \textit{Fnip1} homozygous \textit{Fnip2} double-knockout mice. Tumor developed from cyst wall (Tumor1 of Mouse1, 692 d old) or within the kidney without prominent infiltration (Tumor2 of Mouse1). Cells lining the cyst walls (Cyst1 of Mouse2) were occasionally piled up (Cyst2 of Mouse2, 670 d old). The most frequent histology was the hybrid oncocytic tumor (Tumor1–Tumor3 of Mouse2). Papillary projections from the cyst wall were occasionally observed (Mouse3, 699 d old). (Scale bars: 1 mm, 100 μm, and 20 μm.) (D) Western blotting shows increased Pparc1a and phospho-proteins of the AKT-mTOR pathway in kidney tumors from \textit{Fnip1} \textit{Fnip2} double-knockout mice. GAPDH served as a loading control (n = 7 each; 628–699 d old). (E) Immunostaining of kidney tumors from \textit{Fnip1} \textit{Fnip2} double-knockout mice demonstrate increased phospho-mTOR and pS6R downstream readout of mTOR compared with adjacent normal kidney.
Cell Lines. Fnip1/Fnip2 null MEFs (DKO) were generated by deleting all alleles of Fnip1 and Fnip2 in MEFs extracted from Fnip1<sup>−/−</sup>, Fnip2<sup>−/−</sup> mice, using adenoviral Cre recombinase (17). These MEFs were restored with either wild-type Fnip1 (DKO+Fnip1) or wild-type Fnip2 (DKO+Fnip2), using Tet3G system (Clontech) (32). To eliminate a possible artifact from doxycycline, 0.5 μg/μl doxycycline was added to all lines. Doxycycline-inducible wild-type FLCN-expressing UOK257 (6) was transfected with Silencer Select Predesigned SiRNA (Ambion) for Fnip1: 5′-GCCAUAUGAAGUAGU-Uatt-3′ and Fnip2: 5′-CCACAUGUGAUAUGUatt-3′, using Lipofectamine RNAiMAX (Invitrogen) transfection reagent. This protocol was approved by the National Institutes of Health's Institutional Animal Care and Use Committee and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the National Research Council's "Guide for the Care and Use of Laboratory Animals."

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ATP Measurement. ATP was measured using ATPlite 1step (Perkin-Elmer) according manufacturer’s protocol.

Statistical Analysis. Experimental data are summarized as the mean values ± SD. Statistical analyses were performed using a two-tailed Student t test (SPSS Statistics version 20), and differences were considered to be statistically significant at a value of P < 0.05. Survival curves were obtained using GraphPad Prism version 6.01.

Animal Care. National Cancer Institute-Frederick is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the National Research Council’s “Guide for the Care and Use of Laboratory Animals.”